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Synthesis and evaluation of a new series of substituted acyl(thio)urea and thiadiazolo [2,3-*a*] pyrimidine derivatives as potent inhibitors of influenza virus neuraminidase

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Abstract—A series of substituted acyl(thio)urea and 2*H*-1,2,4-thiadiazolo [2,3-*a*] pyrimidine derivatives were prepared and both of their cell culture and enzymatic activity toward influenza virus were tested. Their in vitro neuraminidase inhibitory activities were in good agreement with the corresponding activities in cultured cells and they were evaluated as potent neuraminidase inhibitors. Of the analogues that demonstrated IC_{50}s < 0.1 μM, **16** and **60** were further investigated as candidates with the most potential for future development. The molecular docking work of the representative compound was described to provide more insight into their mechanism of action and further rationalize the observations of this new series herein, which represents a novel class of highly potent and selective inhibitors of influenza virus.

1. Introduction

Influenza virus commonly known as flu is the contagious etiologic agent that causes an acute respiratory infection, hence it has always been a major threat[1] to human health worldwide and cause for economic costs. Each year about 120 million people in North America, Europe, and Asia are infected, while in the USA alone more than 200,000 patients are admitted to hospitals because of influenza and there are approximately 30,000–40,000 influenza-related deaths.

Currently, the developments of vaccines for influenza virus are of restricted usefulness as they are not susceptible to the high mutability of the virus. Effective chemotherapy for influenza virus is also limited due to newly discovered drug resistance[2] in mutant strains. The obvious choice remains the development of effective drugs, which are not susceptible to mutation. Although the viral replicative cycles has revealed several potential molecular targets (M2 proteins,[3] endonuclease,[4] hemagglutin,[5] and neuraminidase[6]) that can be used for anti-flu drugs design, there are currently only a few licensed drugs available for influenza treatment. M2 inhibitors such as amantadine and rimantadine, which act specifically against influenza A virus by blocking the ion channel of the M2 protein, provide only limited protection due to a narrow spectrum of activity.[7] The only neuraminidase (NA) inhibitors which received FDA approval are zanamivir and oseltamivir. Though zanamivir displays excellent antiviral activity when administered intranasally, it is less effective when delivered systemically. It has very low oral bioavailability and is rapidly eliminated by renal excretion.[8] Oseltamivir is orally active, but it has been reported to cause vomiting and nausea. Thus, there is still a great need to design, screen, and identify new agents for the chemotherapy of influenza virus infection, and research for more effective drugs following systemic administration is a high priority.

During the past decade, thiourea derivatives have been reported effective against HIV[9,10] and to have bactericidal action.[11] Few studies have been done concerning evaluations of substituted acyl(thio)urea and 2*H*-1,2,4-

Keywords: Influenza virus; Neuraminidase inhibitor; Acyl(thio)ureas derivatives; Pyrimidine derivatives; Antiviral activity.
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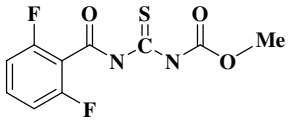
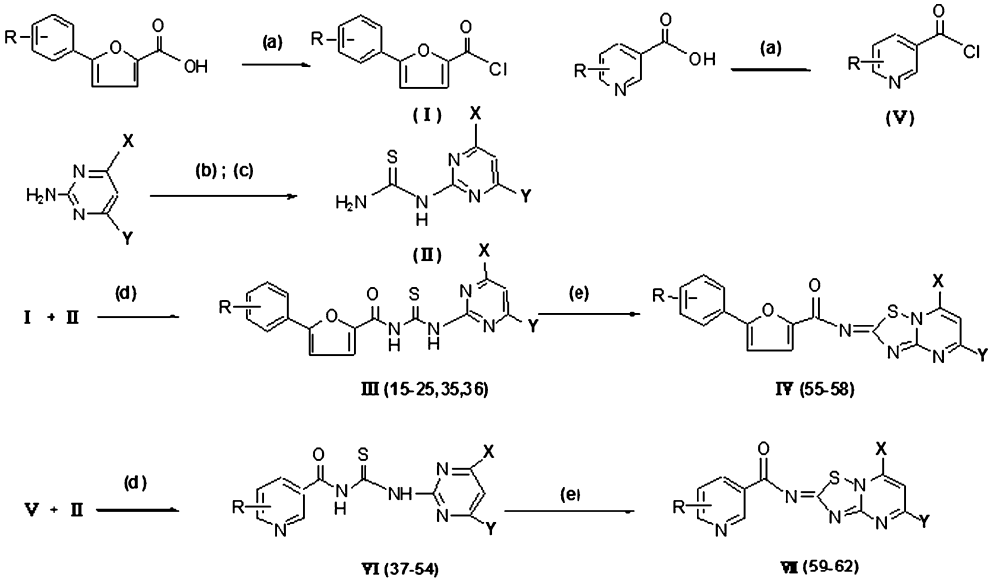


Figure 1. Initial influenza inhibitor, **10**.

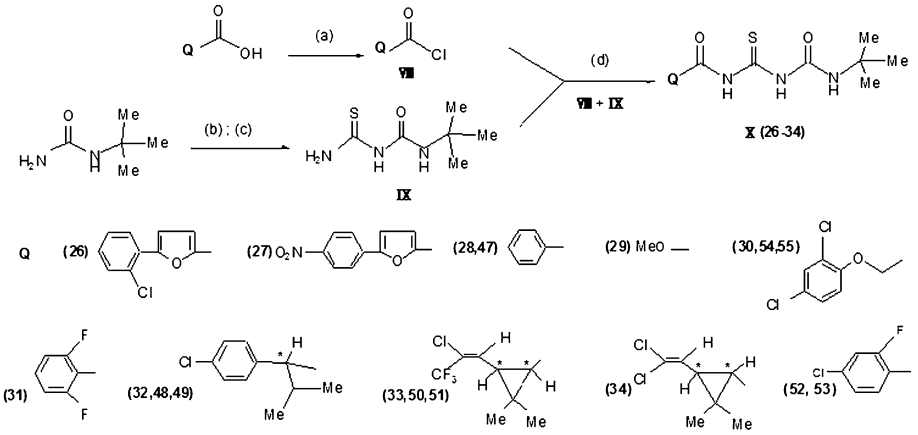
thiadiazolo [2,3-*a*] pyrimidine for their antiviral activity. In our early attempts to screen for activity against different viruses, acyl(thio)urea **10** (Fig. 1) was revealed as a weak inhibitor of influenza virus. As part of the program to maximize this activity and identify more effective influenza virus inhibitors as well as searching for the specific influenza target(s),[12] a new class of substituted acyl(thio)urea and 2*H*-1,2,4-thiadiazolo [2,3-*a*] pyrimidine derivatives (designated SATPs, series **I**–**XII**) were prepared and a highly specific anti-influenza virus activity in cell culture was discovered. Their in vitro inhibitory activities of influenza neuraminidase were also investigated and found to correlate well with their antiviral efficacy in cell culture, thus they were evaluated as effective neuraminidase inhibitors. In this study, we describe further details of our new discovery: the in vitro antiviral properties and neuraminidase inhibitory activities of this new compound series. The docking work was also reported to rationalize the observations herein.

2. Chemistry

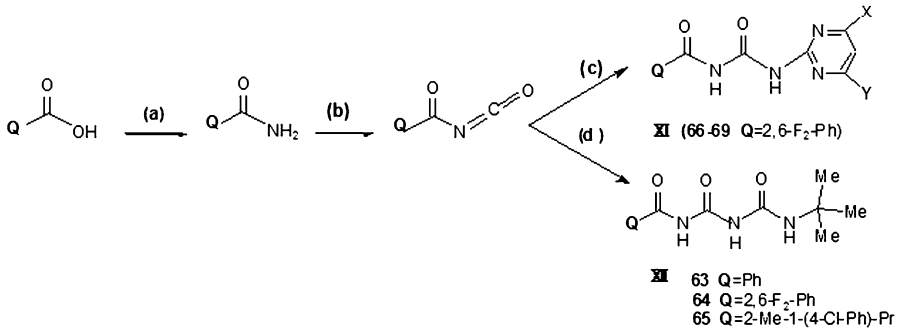
Compounds were synthesized using methods presented in Schemes 1–3. Scheme 1 presents the method used to prepare the reported compound series **III**, **IV**, **VI**, and **VII**. The intermediate *N*′-(4,6-disubstituted pyrimidine-2-yl)-thiourea **II** was obtained by the reaction of a hot aqueous solution of 2-amino-4,6-disubstitued pyrimidine with potassium thiocyanate (KSCN). And 5-aryl-2-furoic acid was allowed to react with $SOCl_2$ to provide the intermediate 5-aryl-2-furoyl chloride **I**. *N*′-(4,6-Disubstituted pyrimidine-2-yl)-*N*-(5-aryl-2-furoyl) thiourea **III** was prepared by the reaction of the intermediate **I** with **II**, followed by oxidizing cyclization via dropwise treatment with bromine as oxidant to produce the target thiadiazolo [2,3-*a*] pyrimidines **IV**.



Scheme 1. Reagents: (a) $SOCl_2$; (b) HCl; (c) KSCN/CH_3CN; (d) Et_3N; (e) Br_2.



Scheme 2. Reagents: (a) $SOCl_2$; (b) HCl; (c) KSCN/CH_3CN; (d) Et_3N.



Scheme 3. Reagents: (a) $NH_3(H_2O)$; (b) $(COCl)_2$; (c) 2-amino-4,6-disubtituted pyrimine; (d) $H_2NCONHC(CH_3)_3$.

The substituted nicotinic acid was acylated with thionyl dichlorideto give substituted nicotinyl chloride **V**.[13,14] The preparation of *N*′-(4, 6-disubstituted pyrimidine-2-yl)-*N*-substituted β-pyridinecarbonyl thiourea **VI** and thiadiazolo [2,3-*a*] pyrimidines **VII** was quite similar to the procedure for the acyl(thio)urea derivation **III** described above.

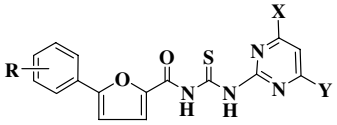
Scheme 2 presents a synthesis of the reported compound series **X**. The intermediate **VIII** (substituted formyl chloride) was prepared by acylation with substituted formic acid using thionyl dichloride. And the intermediate **IX** *N*′-(*tert*-butylaminocarbonyl)-thiourea was synthesized by the reaction of a hot aqueous solution of *tert*-butylurea containing hydrochloric acid with potassium thiocyanate. The target product **X** was synthesized by the reaction of intermediate **VIII** with equal molar of **IX** (Scheme 2) in $(CH_3CH_2O)_3N$ and acetonitrile, and the mixture was refluxed for 3 h. After removal of the volatiles in vacuo, the resulting precipitate was collected by filtration and desiccated. The final compound **X** was recrystallized from acetonitrile.

The method for the synthesis of compounds series **XI** and **XII** is shown in Scheme 3. They are prepared starting from the substituted formic acid, followed by treatment with ammonia liquor and subsequent reaction with $(COCl)_2$. Then the substituted acylisocyanates were converted to the target acylurea derivatives **XI**, **XII** by reacting with 2-amino-4,6-substituted pyrimidines and *tert*-butylurea, respectively.

3. Results and discussion

The SAR of different substituents in the pyrimidine ring is shown in Table 1. Different substituents at the 4- or 6-position in the pyrimidine ring had greatest influence on activity. In this series of 5-aryl-2-furoyl thioureas bearing a substituted pyrimidine ring, there was a large preference for electron-donating groups such as Me, OMe, OEt, and OH in this portion of the molecule (Table 1; **15**–**18**). Substitution by electron-withdrawing groups such as Cl (**19**–**20**) led to poor activity ($IC_{50} > 20$ μM). For instance, the acyl(thio)urea **16** was the most active compound in the series with IC_{50} of 0.08 μM. However, the 4-nitrophenyl substitution did not offer much improvement over 2-chlorophenyl substituted

Table 1. SAR of polysubstituted pyrimidinyl acyl(thio)urea analogues



15-25

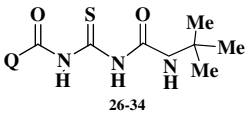
Compound	R	X	Y	IC_{50}[a,b]
15	2-Cl	OEt	Me	1.65
16	2-Cl	OEt	OEt	0.08
17	2-Cl	OH	Me	0.32
18	2-Cl	OMe	OMe	1.77
19	2-Cl	Cl	Cl	14.5
20	2-Cl	Cl	OEt	>20
21	2-Cl	OMe	Me	>20
22	4-NO_2	Cl	Cl	1.66
23	4-NO_2	OEt	OEt	>20
24	4-NO_2	OEt	Me	2.3
25	4-NO_2	OH	Me	0.36

[a] IC_{50} of influenza (H1N1) virus, μM; MTS $IC_{50} > 300$ μM for all compounds. Data are means of three assays.
[b] cf., the cell culture inhibitory activity of zanamivir; IC_{50}, 0.05 μM.

analogues, except for **22** (**22** vs **19**). Other corresponding 4-nitrophenyl derivatives (**23**, **24**) had no appreciable activity toward influenza virus when compared with their 2-chlorophenyl counterparts (**15**, **16**). Interestingly, the 4-nitrophenyl derivative **25** was an exception, which was found to inhibit the influenza virus in vitro at 0.36 μM and showed activity comparable to that of **17**. Nonetheless, substitutions on the pyrimidine ring profoundly affect the ability to inhibiting influenza virus in vitro.

Introduction of the *tert*-butylaminocarbonyl group afforded improved activity. Replacement of polysubstituted pyrimidine by *tert*-butylaminocarbonyl (**26**, **27**) further increased the potency 8- to 10-fold over analogue **19**, **24** (Table 2). With regard to the substituent Q, replacement of 5-aryl-2-furyl by groups such as phenyl or methoxyl (**28**, **29**) resulted in a slight decrease in the potency, while other substituents (**30** ~ **32**) showed activity that was equipotent to that of analogue **26**. Introduction of a specific functional cycloalkyl such as chrysanthemoyl (**33**, **34**) resulted in a significant increase in potency, and analogue **34** was the most potent compound in this series and inhibited influenza virus in vitro at a concentration of 0.26 μM.

Table 2. *tert*-Butylaminocarbonyl acyl(thio)urea analogue SAR



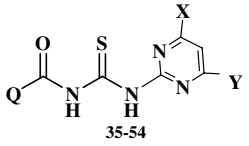
26-34

Compound	Q	IC_{50}[d]
26	5-(2-Cl–Ph)–2-Furyl	1.42
27	5-(4-NO_2–Ph)–2-Furyl	1.30
28	Ph	1.79
29	OMe	1.83
30	(2,4-Cl_2–Ph)–OCH_2	1.67
31	2,6-F_2–Ph	1.43
32[a] **S**-(+)	2-Me-1-(4-Cl–Ph)–Pr	1.35
33[a] *cis*-(−)	*CFPC[b]	0.51
34[a] *trans*-(−)	*DCPC[c]	0.26

[a] Compounds **32**–**34** are pure enantiomers as indicated.
[b] *CFPC, 3-(2-chloro-3,3,3-trifluoropropenyl)-2,2-dimethyl cyclopropyl.
[c] *DCPC, 3-(2,2-dichloro ethenyl)-2,2-dimethyl cyclopropyl.
[d] IC_{50} of influenza (H1N1) virus, μM.

Other acyl(thio)ureas bearing a substituted pyrimidine ring without 5-aryl-2-furyl were synthesized and resulted in lowered potency (Table 3). This demonstrated a large advantage in activity of the existence of a five-membered ring (furan) over corresponding analogues without the furan ring in most instances, and this trend continued for other series of compounds. The compounds containing furan ring (**35**, **36**) were 15-fold more potent than the corresponding 6-chloro-3-pyridyl analogues (**37**, **38**), which showed no activity up to 20 μM. However, the only analogue with no furan ring that retained good activity in this series was the test compound **44**, although it was 2-fold less potent than the corresponding furyl substituted analogue **36**. This result suggests that a furan ring at this position provides improved activity and should be included in the rational design of new influenza virus inhibitors. Variation of the **X**, **Y** substituents in the pyrimidine ring (**39**–**43**) and other analogues with different aromatic substituents as Q (**45**, **46**, and **52**–**54**) was also examined. They did not achieve good efficiency against influenza virus except for **53**. Additionally, substitution of chloro- at the 2- or 6-position in the pyridine ring was not a major determinant in activity (**39** vs **42**) in this series. Analogues sharing the same functional cycloalkyl substituents (**50**, **51**) as **33** show similar inhibitory activity and replacement of methyl by methoxy in the pyrimidine ring did increase the potency slightly (**50** vs **51**) but was not significant. Investigation of ethoxy substituents at the C-4, 6 positions of pyrimidine ring produced analogue **49** that has significant improvement in the potency, while analogue with substituents as chlorine (**48**) was again not encouraging.

Table 3. SAR of aryl and chrysanthemoyl Q groups



35-54

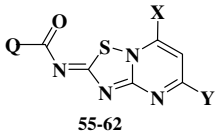
Compound	Q	X	Y	IC_{50}[a]
35	5-(4-NO_2–Ph)–2-Furyl	OMe	Me	1.22
36	5-(2-Cl–Ph)–2-Furyl	OMe	Cl	1.29
37	6-Cl-3-py	OMe	Me	>20
38	6-Cl-3-py	OMe	Cl	>20
39	6-Cl-3-py	OEt	OEt	>20
40	6-Cl-3-py	Me	OH	8.58
41	2-Cl-3-py	Me	Me	7.19
42	2-Cl-3-py	OEt	OEt	>20
43	2-Cl-3-py	OEt	Cl	>20
44	2-Cl-3-py	OMe	Cl	2.59
45	3-py	OMe	OMe	>20
46	5,6-Cl_2-3-Py	OMe	OMe	18.5
47	Ph	Me	Me	2.1
48	2-Me-1-(4-Cl–Ph)–Pr	Cl	Cl	>20
49	2-Me-1-(4-Cl–Ph)–Pr	OEt	OEt	0.31
50	*CFPC[b]	OMe	OMe	0.97
51	*CFPC[b]	Me	Me	0.58
52	2-F-4-Cl–Ph	Me	Me	1.36
53	2-F-4-Cl-Ph	OMe	Cl	5.1
54	(2,4-Cl_2–Ph)–OCH_2	OMe	OMe	1.89

[a] IC_{50} of influenza (H1N1) virus, μM.
[b] *CFPC, 3-(2-chloro-3,3,3-trifluropropenyl)-2,2-dimethyl cyclopropyl.

The formation of 2*H*-1, 2, 4-thiadiazolo [2,3-*a*] pyrimidine ring had relatively small effect on potency against influenza virus (Table 4, **56**, **57** vs **25**, **23**). However, compounds **58** and **60** in this series inhibited the virus with an IC_{50} of 0.35 and 0.09 μM, respectively. Compound **58** showed improved potency over the corresponding un-ringed analogues such as **15**. The high potency of **60** bearing the same ethoxy substituents as the active analogues **16**, **49** is further support of a preference for electron-donating groups such as ethoxy in their activity.

The necessity for a thiourea functionality (Table 5) was also investigated. The corresponding ureas (**63**–**69**) were found to be less effective than their thiourea counterparts were. Replacement of the thiourea bridge with urea diminished or eliminated activity altogether in most cases. For instance, **64**, **65** had a 10-fold reduction in activity compared to **31**, **32**. In this respect, it follows that the thiourea bridge is an important contribution to inhibitory activity against influenza virus.

Table 4. Effect of 2*H*-1,2,4-thiadiazolo [2,3-*a*] pyrimidine ring on inhibition of influenza virus

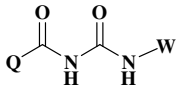


55-62

Compound	Q	X	Y	IC_{50}[a]
55	(2,4-Cl_2–Ph)–OCH_2	OMe	OMe	>20
56	5-(4-NO_2–Ph)–2-Furyl	Me	OH	0.67
57	5-(4-NO_2–Ph)–2-Furyl	OEt	OEt	5.25
58	5-(2-Cl–Ph)–2-Furyl	OEt	Me	0.35
59	6-Cl-3-py	OMe	Cl	9.32
60	6-Cl-3-py	OEt	OEt	0.09
61	2-Cl-3-py	OMe	OMe	2.41
62	3-py	OMe	OMe	>20

[a] IC_{50} of influenza (H1N1) virus, μM.

Table 5. Thiourea versus urea comparison



63-69

Compound	Q	W	IC_{50}[a]
63	Ph	CONH(*t*-Bu)	>20
64	2,6-F_2–Ph	CONH(*t*-Bu)	14.1
65	2-Me-1-(4-Cl–Ph)–Pr	CONH(*t*-Bu)	15.5
66	2,6-F_2–Ph	4,6-$(Me)_2$–2-Pym[b]	>20
67	2,6-F_2–Ph	4,6-$(OMe)_2$–2-Pym	>20
68	2,6-F_2–Ph	4-Cl-6-(OMe)–2-Pym	15.9
69	2,6-F_2–Ph	4-Cl-6-Me–2-Pym	>20

[a] IC_{50} of influenza (H1N1) virus, μM.
[b] Pym, pyrimidinyl.

It was noted that the *tert*-butylacyl(thio)urea analogues have improved water solubility while retaining good lipophilicity, which may contribute partially to the increase in its anti-viral potency. Although some of the other **SATP**s have a relative low solubility (<10 mg/mL at room temperature), their high lipophilicity is expected to lead to an efficient penetration of the **SATP**s through cellular membranes and biological barriers. This property may be advantageous for this novel class of influenza inhibitors, as it may facilitate the uptake of the lipophilic **SATP**s into cells or biological compartments where the virus accumulates.

Mechanistic investigations into the mode of inhibition led to enzymatic studies for the activity of this set of compounds toward influenza neuraminidase, which is an essential surface glycoprotein that is required for the release of newly formed virus. It was found that (Table 6) analogues which were highly active inhibitors in cell culture also showed good inhibitory activities in this enzymatic studies, and the concentrations at which the 50% inhibition of neuraminidase activity was achieved were in the same range as those observed in their antiviral evaluations in cultured cells. Besides, Compound **60** (IC_{50} = 0.05 μM) was again encouraging and it shows the highest inhibitory activity toward the influenza neuraminidase. The fact that the neuraminidase inhibitory activities were in good agreement with the corresponding antiviral efficacy in cultured cells suggests that the **SATP**s belongs to a novel class of NA inhibitors.

For comparison, both cell culture and enzymatic activity of zanamivir, which is used as a positive control, were also evaluated in the same system with all reported compounds. It was noted that zanamivir exerted higher potency than most of **SATP**s in inhibiting the enzymatic activity. However, the influenza strain tested in these assays seemed to be insensitive to these well-known NA inhibitors, as neither of zanamivir's cell culture (Table 1) nor enzymatic activity (Table 6) was satisfying when compared with the data that had been previously reported in 1998,[17] which also implies an increasing need for developing novel flu inhibitors with new structures.

In general, the NA inhibitors known as NAI do not inhibit virus replication but do prevent the release and spread of the virus from infected cells, and effectively retard its propagation. They have a broader antiviral spectrum, better tolerance, proven efficacy in reducing respiratory events, and less potential for rapid emergence of clinically important resistance than is seen with the M2 inhibitors. Therefore, the NAIs represent an important advance over the M2 inhibitors in influenza therapy. Although not currently approved for prevention in some countries, they do seem to be effective for chemoprophylaxis and could be used for long-term protection of those not responding to vaccine, or ineffective due to antigenically novel viruses. In this regard, this new compound series evaluated herein as neuraminidase inhibitors has the potential to become the candidates for future development of new effective drugs.

Table 6. In vitro influenza neuraminidase inhibitory activity of the representative compounds of PAFHs

Compound	IC_{50}[a] (μM)	Compound	IC_{50}[a,b] (μM)
15	1.3	**40**	11.0
16	0.06	**41**	9.5
17	0.21	**44**	2.8
18	1.3	**46**	n.a[c]
19	15.9	**47**	1.6
22	1.2	**49**	0.27
25	0.25	**50**	0.85
26	1.2	**51**	0.43
27	1.1	**52**	4.2
28	1.5	**53**	1.1
29	1.5	**54**	1.6
30	1.4	**56**	0.69
31	1.2	**57**	4.2
32	1.3	**58**	0.26
33	0.46	**59**	12.2
34	0.19	**60**	0.05
35	1.3	**61**	2.3
36	1.3	**65**	18.7

[a] The concentration of inhibitor that requires to reduce the NA activity by 50%.
[b] cf., neuraminidase inhibitory activity of zanamivir; IC_{50}, 0.02 μM.
[c] n.a., not active in the concentration range tested (0.01–20 μM), other compounds which have IC_{50} value > 20 μM are not shown.

3.1. Molecular docking

As an efficient approach for investigating protein–ligand interactions, molecular docking plays a key role in rational drug design,[15] especially when the crystal structure of the protein has been published. Since the crystal structure of influenza virus neuraminidase complexed with zanamivir is available, the interactions in this crystal complex with the corresponding entry code 1a4g in PDB were first explored. For further SAR analysis, molecular docking has been performed to investigate the key interactions responsible for the high potency of this class of inhibitors as well as the binding mode. FlexX, which is a reliable method in complex validation tests among various docking algorithms,[16] was chosen as the docking protocol. FlexX scores were employed for evaluation, since they consist of a variety of score terms as well as the relative affinity between the enzyme and inhibitors. Quite a few analogues reported herein, including the most promising compound **60**, were docked to neuraminidase. Since most of the active

compounds shared quite similar binding modes, the docking results of the representative analogues are discussed and shown in Figure 3. Besides, their total FlexX scores are also provided in Table 7 to quantify and investigate the docking results.

The crystal structure of neuraminidase-zanamivir complex (Fig. 2) demonstrated the pattern of protein–ligand interactions, which consist of strong charge-charge- and charge-partial charge-based hydrogen bonds. The carboxylic acid of zanamivir makes charge-charge-based hydrogen bonds to a cluster of three arginine residues, 115, 291, and 373, including a planar salt bridge to 373. In addition to the hydrogen bonds from the terminal hydroxyl groups of the glycerol moiety to the carboxyl of Tyr 408, there is an electrostatic interaction between the guanidino substituent and Asp 148. These interactions were suggested to account for its inhibitory potency and selectivity on neuraminidase[17] from influenza virus.

With respect to compound **60**, the top 10 molecular docking results of FlexX were analyzed, and they bound in a closely similar manner to influenza neuraminidase with their scaffold well suited to interact with the active site of the neuraminidase. Based on the comparison with the crystal complex mentioned above, though **60** shared different structural properties with those of zanamivir, they seemed to bear a similar binding mode (Fig. 3a). The acyl backbone of the ligand also makes charge-charge-based hydrogen bonds to the guanidino groups of arginine residues 115, 291, and 373 which are colored in green in the binding cavity. The pyridine ring of **60** does not appear to make strong interactions to any active-site residues, although there may be a weak electrostatic interaction between the ring nitrogen and Asp 148. In addition, the acyl nitrogen on the backbone of **60** forms hydrogen bonds to the hydroxyl of Tyr 408 colored in yellow, while the oxethyl fragment improves the activity by making a targeted hydrogen bond with Asn 293 colored in purple. These hydrogen bonds may further strengthen the binding of the ligand, resulting in a strong network of hydrogen-bonding interactions.

Table 7. The total FlexX scores of the representative analogues

Compound	Total FlexX score
19	−22.56
22	−33.62
28	−27.60
60	−36.23

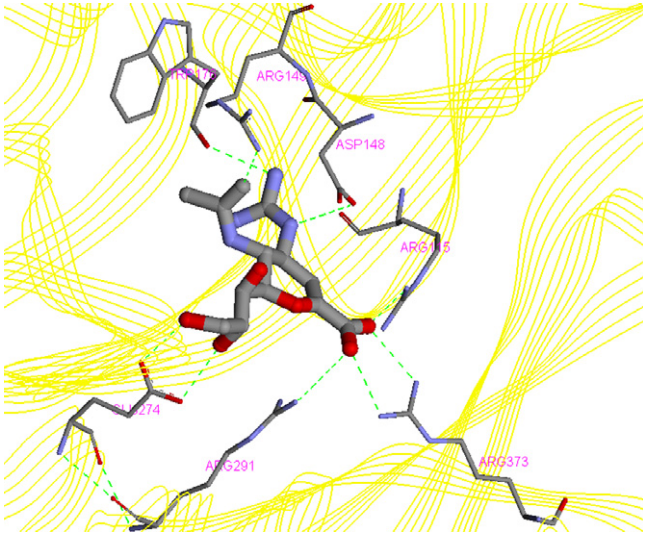


Figure 2. Crystal structure of zanamivir bound in the active site of influenza neuraminidase. Key amino acid residues forming the subsite are highlighted and the major protein–ligand salt bridges, hydrogen-bonding interactions are shown using Sybyl 6.9.

The docking results of the other three representative analogues are shown in Figures 3b–d. Most of the active compounds shared quite similar binding mode with **60**. Furthermore, the total FlexX score of compounds **60**, **28**, **22**, and **19** (Table 7) are in good agreement with previous enzymatic inhibitory activity, since a lower score value may indicate a more energy favorable state and more strong affinity for the binding complex. It was noted that compound **19** (Fig. 3d) did not bind to the pocket at the entrance of the binding cavity surrounded by the three arginine cluster, which is in accordance with its relatively low neuraminidase inhibitory activity.

The oxyethyl groups of **60** occupy a small pocket at the entrance of the binding cavity, which may prevent the substrate of NA from entering into the active site. This may explain the high inhibitory activity of compound **60** toward the influenza neuraminidase. Moreover, this docking result further demonstrates the importance of these approaches to understanding the interactions between the enzyme and **SATP**s, which may provide more insight into the mechanism of action of these newly discovered inhibitors as well as some guidelines for structural optimization in drug design.

4. Conclusion

In this paper, we have described the synthesis of a series of substituted acyl(thio)urea and 2*H*-1,2,4-thiadiazolo [2,3-*a*] pyrimidine. Evaluation of their in vitro antiviral activity revealed compounds with high potent cellular activity against wild-type influenza virus in cultured MDCK cells. Meanwhile, the neuraminidase inhibitory activities of these compounds correlate well with their in vitro antiviral efficacy in cell culture, which reveals that their specific influenza target was the neuraminidase from influenza virus. Analogues **16** and **60** (Fig. 4) inhibited the influenza virus with an IC_{50} of 0.08 and 0.09 μM, respectively, and these novel inhibitors were investigated as candidate compounds with the most potential for future development. This research leads to a better understanding of SAR of influenza virus inhibitors and thereby provides some insight into the rational design of anti-flu agents and the discovery of new effective drugs.

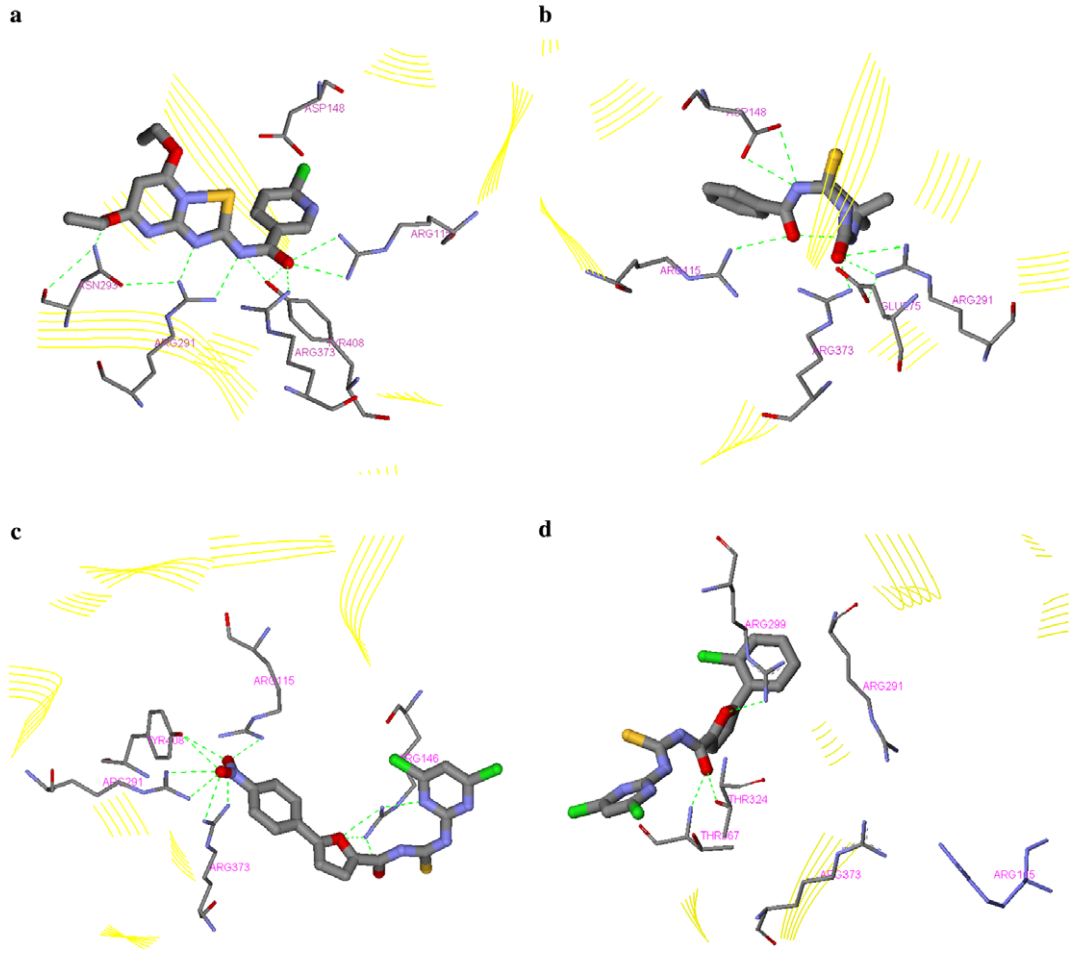
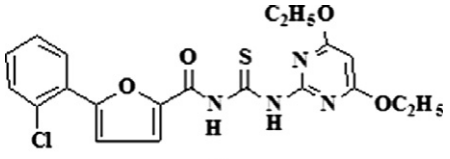
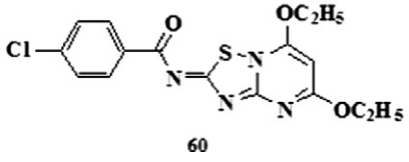


Figure 3. Stereo view showing the binding of representative compounds to the active site of influenza neuraminidase. (a) The hydrogen-bonding interaction between compound **60** and Arg 115, 291, 373 (atoms colored green) is illustrated by dashed lines, as are other associated hydrogen bonds (see text); other amino acid residues near the active site are shown in line ribbons. (b–d) The docking poses and interactions of compounds **28**, **22**, and **19**, respectively.



16



60

Figure 4. Advanced potential development candidates **16**, **60**.

5. Experimental

5.1. In vitro assay in MDCK cells

The MDCK cells were grown in 96-well microtiter plates in minimal essential medium (MEM) containing 10% FBS; influenza Beijing/30/95 virus (H1N1) were amplified and titrated in MDCK cells at 50 plaque-forming units (PFU)/well. Each compound was serially diluted and tested against the virus to calculate its IC_{50} (Tables 1–5) and all values are means of at least three independent experiments on different days. Additionally, general cytotoxicity was measured using the MTS cellular toxicity assay[18,19] to distinguish between specific anti-viral activity and non-specific host cell toxicity. All the compounds reported herein have MTS IC_{50} values of >300 μM and are considered to be noncytotoxic (these data are not included in the tables).

5.2. Enzyme assay

The in vitro enzyme assay is based on the method reported by Vonitzstein et al.[20] with some improvence. The neuraminidase from the H1N1 strain of influenza was obtained by the method described by Tai et al.[21] Values for the IC_{50} were measured via a spectrofluorometric technique that uses 2′-(4-methylumbelliferyl)-α-D-acetylneuraminic acid as substrate. This substrate was cleaved by neuraminidase to yield a fluorescent product, which can be quantified. The assay mixture contained inhibitors at various concentrations and enzyme in 33 mM MES (2-(*N*-morpholino) ethanesulfonic

acid) buffer, 4 mM $CaCl_2$ at pH 6.5 (total volume = 100 μL). There action was started by the addition of 10 μL of the substrate to a final concentration of 20 μM. After 15 min at 37 °C, 150 μL of 14 mM NaOH/83% ethanol was added to 0.1 mL of the reaction mixture to terminate the reaction. A blank was run with the same substrate solution with no enzyme. Fluorescence was read using an Aminco–Bowman fluorescence spectrophotometer (excitation, 355 nm, emission, 460 nm) and substrate blanks were subtracted from the sample readings. The IC_{50} was calculated by plotting percent inhibition versus the inhibitor concentration, and determination of each point was performed in triplicate.

5.3. General chemistry methods

All solvents and reagents were commercially available and used as received. Melting points were obtained in open capillary tubes in a 8100 digital melting point apparatus and are uncorrected. IR spectra were run as KBr pellets on a Bruker Eqinox 55 FTIR spectrometer. 1 H NMR spectra were recorded on a Varian-XL-400 nuclear magnetic resonance apparatus, Elemental analysis was performed on the Germany Elementar Vario EL elemental analyzer.

5.4. Molecular docking procedure

All calculations were carried out on a R14000 SGI Fuel workstation using the software package SYBYL version 6.9 (Tripos, St. Louis, MO, USA). FlexX 1.11.1 within SYBYL package was employed to explore the interaction between the ligand and enzyme. The crystal structure of influenza virus neuraminidase complexed with zanamivir was retrieved from PDB with corresponding entry code 1a4g. Then the protein was prepared by removing heteroatoms and water molecules and adding all hydrogen atoms. The active site of 1a4 g was defined as residues with at least one atom within a radius of 9 Å from any atom of zanamivir. Then all compounds were sketched using sybyl with all hydrogen atoms added and Gasteiger-Huckel[22] charges were used to parameterize the compounds. Furthermore, their conformers with low energy were ensured by RANDOM searches available in SYBYL. Then the compounds were docked to neuraminidase from influenza virus by FlexX facilities. FlexX scoring function was employed to evaluate the docking pose of the compounds. The top 10 docking results of each compound were used for further discussion.

5.5. Physical data for two key compounds: *N*′-(4, 6-diethoxylpyrimidin-2-yl)-*N*-[5-(2-chlorophenyl)-2-furoyl]thiourea (compound 16)

Yield: 86%, mp: 175–177 °C; IR(KBr plate, cm^{-1}): 1632(C═O), 3195 (N═H), 1235(C═S) ^1H NMR (DMSO-d_6): δH_{ppm} 7.24–7.86 (m, 6H, Ar–H), 6.15 (s, 1H, pyrimidine-5-H), 12.08 (s, 1H, N′–H), 12.46 (s, 1H, N′–H), 1.22 (t, 6H, CH_3), 4.08 (q, 4H, OCH_2) Anal. Calcd (%) for $C_{20}H_{19}ClN_4O_4S$: C, 53.75; H, 4.26; N, 12.54. Found: C, 53.64; H, 4.35; N, 12.41.

5.6. 5,7-Diethoxyl-2-(6-chloro-3-pyridinecarbonylimino)-2*H*-1,2,4-thiadiazolo[2, 3-*a*]pyrimidine (compound 50)

Yield: 72%, mp: 176–178 °C; IR (KBr plate, cm^{-1}): 1690 (C═O), 1630 (C═N); ^1H NMR (DMSO-d_6): δH_{ppm} 7.60–8.95 (m, 3H, pyridine-H), 5.65 (s, 1H, pyrimidine-5-H), 1.16 (t, 3H, CH_3), 4.18 (q, 2H, OCH_2); Anal. Calcd (%) for $C_{20}H_{17}ClN_4O_4S$: C, 47.43; H, 3.69; N, 18.45. Found: C, 47.51; H, 3.60; N, 18.36.

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