Design, Synthesis, and Preliminary Evaluation of New Pyrrolidine Derivatives as Neuraminidase Inhibitors

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Abstract: A series of pyrrolidine derivatives were designed and synthesized in good yields starting from commercially available 4-hydroxy-*L*-proline using a suitable synthetic strategy. And their ability to inhibit neuraminidase was evaluated. These compounds showed potent inhibitory activity against influenza A (H3N2) neuraminidase. Within this series, four compounds, **6e**, **9c**, **9f** and **10e**, have the good potency (IC_{50}=1.56~2.40μM) which is compared to the NA inhibitor oseltamivir (IC_{50}=1.06μM), and could be used as lead compound in the future.

Keywords: Influenza virus, Neuraminidase, inhibitor, pyrrolidine derivatives.

1. INTRODUCTION

Influenza is an acute viral infection of the upper respiratory tract that can affect millions of people every year [1]. The antiviral drugs amantadine and rimantadine are selective for influenza A viruses and their clinical use has been limited by side effects and the rapid emergence of resistant viral strains. Vaccine development has been partially successful in the control of influenza epidemics due to the highly variable mutation of influenza virus. Effective and safe anti-influenza therapeutics are lacking which makes anti-influenza a high-priority and attractive area of drug discovery.

Neuraminidase (NA) is one of the two glycoproteins expressed on the influenza virus surface. The catalytic activity of NA is essential for influenza virus replication and infectivity, so it has been considered a suitable target for designing agents against influenza viruses. Despite the homology identity of NA in different strains is only about 30%, the catalytic site of neuraminidase in all influenza A and B virus is completely conserved [2]. Therefore, NA has been regarded as an attractive target for antiviral drug development. Now, two NA inhibitors, zanamivir and oseltamivir, have been confirmed as effective and safe for the treatment of influenza. Recently, there have been significant efforts in the design and synthesis of inhibitors of influenza NA as potential therapeutics for influenza virus infection.

X-ray crystal structures of complexes of NA with known five- and six-membered ring inhibitors revealed that potent inhibition of the enzyme is determined by the relative positions of the interacting inhibitor substituents (carboxylate, hydrophobic side chain, acetamido, hydroxyl and amino) rather than by the absolute position of the central ring [3]. This led us to design potential NA inhibitors in which the pyrrolidine ring served as a scaffold for substituents that would interact with the active site of NA.

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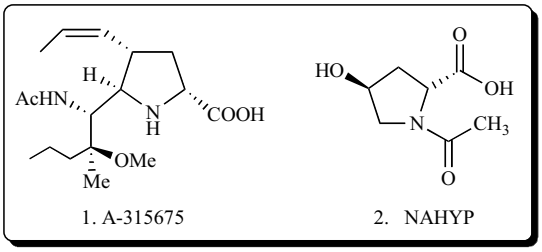
Currently, several pyrrolidine compounds have been found to possess potent NA inhibitory activities [4,5]. For example, A-315675 [5] (**1**) is highly active in cell culture against a variety of strains of influenza A and B viruses. This urges us to developing new NA inhibitors based on pyrrolidine derivatives which contain different substituents (carboxylate, guanidino, acetamino, alkyl) to interact with the four binding pockets of the NA active site.



1. A-315675 2. NAHYP

In our previous work, the 4-hydroxy-*L*-proline has been used to prepare a series of pyrrolidine derivatives as matrix metalloproteinases (MMPs) and aminopeptidase-N (APN) inhibitors [6,7]. We firstly screened all the pyrrolidine derivatives in our compound library including not only the target compounds and intermidates we synthesized before, but also some commercially available compounds. The pharmacological result showed that NAHYP, one anti-inflammation compound (**2**, oxaceprol), exhibited modest activity against influenza virus A (H_3N_2) neuraminidase (IC_{50}=48.73μM) and could be used as lead compounds in further.

In order to improve the affinity of lead compounds, we optimized the structure of NAHYP with following chemical modification: (i) N-acyl group in pyrrolidine ring was changed to other Boc protected or free amino acid residues; (ii) 2-carboxylic acid can be kept or converted to other derivatives such as methyl ester or hydroxymate; (iii) keep hydroxy in 4-position or converted to free amino group.

2. CHEMISTRY

The synthesis of pyrrolidine derivatives possessing NA inhibitory activities was shown in Scheme **1**. The starting

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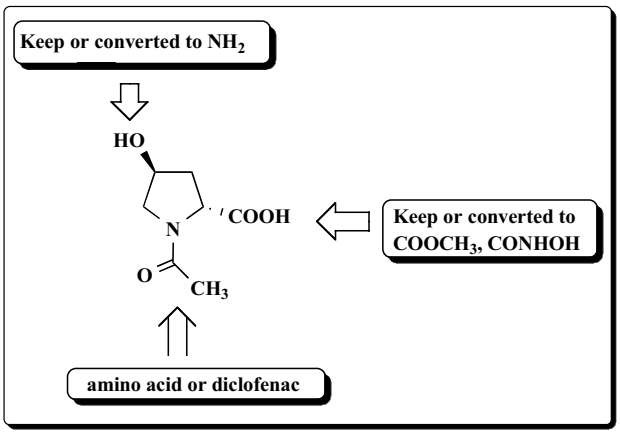


materials, Boc-protected amino acids (**3**) and the 4-hydroxy-*L*-proline methyl ester hydrochloride (**4**), were prepared according to literature [6]. The Boc-amino acids (**3**) were activated with DCC and HOBt and then coupling with compound **4** to yield **5a-10a**. The methyl ester **5a-10a** was then hydrolyzed with $NaOH/H_2O$ or treated with NH_2OK to generate carboxylic acids **5b-10b** or hydroximic acids **5c-10c**.

4-amino-pyrrolidine derivatives were prepared from intermediate **5a-10a.** Firstly, the hydroxyl group of **5a-10a** was converted to mesyl group by methanesulfonate, and then reacted with sodium azide to generate configuration inversed azide. Amino derivatives **5d-10d** can be synthesized by hydrogenation using Pd-$CaCO_3$. The methyl ester **5d-10d** can be transformed to corresponding carboxylic acids **5e-10e** and hydroximic acids **5f-10f** as the methods mentioned before.

The Boc protecting group can easily removed with 3M HCl in ethyl acetate to give hydrochloride salts of pyrrolidine derivatives **5g-10g**. Finally, the amino group was acetylated with acetic anhydride to yield **5h-10h**.

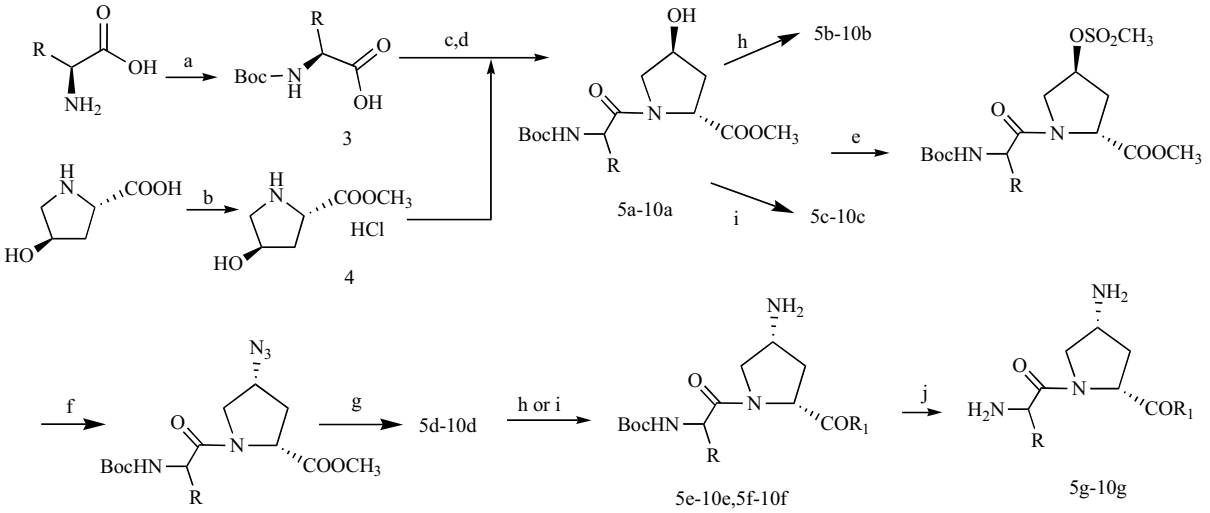
3. RESULTS

The influenza NA inhibitory activities of the compounds were evaluated in an enzymatic assay reported by Guanhua Du[8]. The results of the four most potent compounds are contained in Table **1**. The most effective new compound **10e** was further evaluated for its ability to inhibit viral replication in Madine Darby canine kidney (MDCK) cells. The NA from H3N2 strain of type A influenza was obtained by the method described by Laver [9]. Values for the IC_{50} were measured *via* a spectrofluorometric technique that uses 2'-(4-methylumbelliferyl)-a-D-acetylneuraminic acid (MUNANA) as substrate. Cleavage of this substrate by NA produces a fluorescent product, which can emit an emission wavelength of 460nm with an excitation wavelength of 355nm. The intensity of fluorescence can reflect the activity of NA sensitively. The cytotoxicity and antiviral activity of Compound **10e** in MDCK cell cultures are shown in Table **2**.

From the tables, we found that Compound **10e** with NH_2 group and methionine as hydrophobic side chain showed the best inhibitory activity (IC_{50}=1.56μM). Two compounds containing isoleucine (**9c** and **9f**) exhibited good activities (2.10~2.15μM).

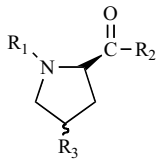
4. DISCUSSION AND CONCLUSION

From the Table **2**, we can see that one of the compounds (**10e**) exhibited some specific activity against influenza A (H3N2) (IC_{50}=3.67μM) which was comparable with the marketed NA inhibitor oseltamivir, and it has a significant selectivity for influenza A over influenza B NA. Although the structures of the catalytic sites in influenza A and B NA appear to be identical, compound (**10e)** is highly selective for influenza A NA. This observation is consistent with earlier reports for NA inhibitors that induce a conformational change in Glu278 upon ligand bind, since this conformational change in influenza B NA is energetically unfavorable



Scheme 1. Reagents and Conditions: (a) $(Boc)_2O$, $NaOH/H_2O$, THF; (b) MeOH, HCl; (c) DCC, HOBt, THF, 0° C; (d) NMM, THF; (e) MsCl, Et_3N, DCM; (f) NaN_3, DMF, 65°C; (g) 5% Pd-$CaCO_3$, H_2, MeOH; (h) R=OOH, MeOH, $NaOH/H_2O$; (i) R=ONHOH, NH_2OK, MeOH; (j) HCl/EtOAc, EtOAc.

Table 1. The Structure and *In Vitro* Inhibitory Activities of Compounds Against NA



Compound	R_1	R_2	R_3	m.p.(℃)	CLogP	IC_{50}(μ g/ml)
6e	Boc-Phe	OH	NH_2	175-176	-0.1341	2.40
9c	Boc-Ile	NHOH	OH	94-95	0.9124	2.15
9f	Boc-Ile	NHOH	NH_2	116-117	0.9894	2.10
10e	Boc-Met	OH	NH_2	153-154	-1.4041	1.56

Table 2. Cytotoxicity and Activity of Compound 10e in MDCK Cell Cultures

Compound	Minimum cytotoxic concentration[a] (μg/ml)	IC_{50} (μM)			
		Influenza A (H3N2)		Influenza B	
		Visual CPE score	MTS	Visual CPE score	MTS
10e	100	8.67	3.67	>100	>100
oseltamivir	>50	1.23	1.01	50	50
BCX-1812	10	3.70	6.00	3.70	6.05
amantadine	>50	3.70	5.21	>50	>50

[a]Required to cause a microscopically detectable alteration of normal cell morphology.

[10]. Evaluation of compound (**10e)** against a selection of NA from other influenza A and B strains suggest that this selectivity for influenza A is general. Structural and computational studies to rationalise the potent activity and specificity of the compound will be proceeded further. This is by itself noteworthy for qualification of this compound as a potential "anti-flu"drug.

We selected target **10e** to make the FlexX Docking utilizing SYBYL 7.0 of TRIPOS Company (Fig. **1**). The binding of compound **10e** in the active site of NA was shown in Fig. (**1**), and we found that the $-CO_2H$ or –CONHOH group of the target compounds interacts with the positively charged site of the NA active site, the exocyclic OH or NH_2 group binds to the negatively charged site, and the Boc group interacts with the hydrophobic site *via* one of the methyl groups. The hydrophobic side chain occupied the hydrophobic pocket of the active site.

In summary, our studies have discovered a new series of pyrrolidine derivatives that have potent NA inhibitory activity. We find that the compounds with ester group have reduced inhibitory potency in comparison to the corresponding compounds with carboxy group or hydroximic acid group. It seemed likely that compounds containing a positively charged amino group rather than a hydroxyl group might be more effective against NA. We reported a more convenient and economical method of the synthesis of pyrrolidine NA inhibitors. Compared to the other research, 4-hydroxy-*L*-proline we used appeared to be an ideal starting material because of its low cost and commercial abundance. Establishing a consistent binding mode was critical to predictive structure-based drug design and discovering potent compounds in the nanomolar range that would potentially be useful for antiviral therapy. The compounds we have got all showed potent NA inhibitory activity, and this finding could be used to design further influenza NA inhibitors.

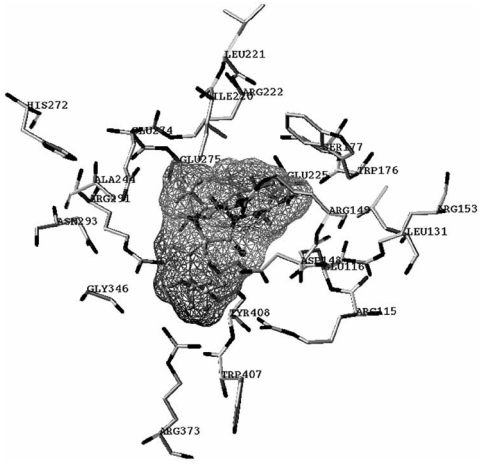


Fig. (1). The complex of neuraminidase with compound **10e**.

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