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# Identification, Synthesis, and Evaluation of New Neuraminidase Inhibitors

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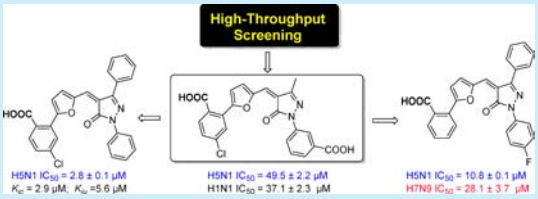
Supporting Information

**ABSTRACT:** High-throughput screening was performed on ~6800 compounds to identify **KR-72039** as an inhibitor of H1N1 and H5N1 neuraminidases (NAs). Structure−activity relationship studies led to **3e**, which inhibited H5N1 NA with an $IC_{50}$ of 2.8 $\mu$M and blocked viral replication. Docking analysis shows that compounds bind to loop-430 around the NA active site. Compound **3l** additionally inhibited H7N9 NA, making it a dual inhibitor of N1- and N2-type NAs.




Avian influenza has sporadically caused pandemic outbreaks. In early 2003, there was an AI H5N1 outbreak in South and Southeast Asia[1] and, more recently, the emergence of the novel AI strain H7N9 in March 2013.[2] Such a sporadic upsurge continues to pose a serious health threat. Unlike the highly pathogenic AI H1N1 virus, H5N1 and H7N9 viruses are less likely to undergo interhuman transmission. However, if the H5N1 virus acquires the ability for interhuman transmission, by either reassortment or mutations as demonstrated in laboratories recently, it would lead to a pandemic that could be much worse than H1N1.[3−7] H7N9 infection could go unnoticed until it reaches humans due to its low pathogenicity in birds.[8] Such a deceptive transmission could be more perilous and has already claimed 44 lives.[9]

Vaccines, as therapeutic agents, suffer support challenges during pandemics which include a longer production time, shorter shelf lives, and the need for annual validation due to continual antigenic variance.[10] Marketed chemotherapeutics targeting M2 ion channels have become obsolete in terms of treating H5N1 and H7N9 infections owing to resistance, thus leaving neuraminidase inhibitors as the only choice.[11,12] Neuraminidase (NA, EC 3.2.1.18) facilitates the detachment of the viral scions from the infected host cell surface in the final step of viral replication and thus serves as the target for anti-influenza drugs oseltamivir and zanamivir. However, the frequent usage of these drugs has led to the development of resistance, for example, to oseltamivir by H5N1 due to H274Y mutation.[13,14] Recently, the outbreak of H7N9 in China[15,16] and the emergence of A/Shanghai/1/2013 H7N9 virus with R292K mutation without the loss of *in vivo* virulence or transmissibility has caused a serious concern because the existing drug treatment, oseltamivir, has become ineffective.[17,18]

Although loop-150 has been considered an ideal region for exploring the further design of the transition-state analogues,[19−21] so far no fruitful results against drug-resistant mutant NAs have been obtained. This necessitates the need to have a newer scaffold with the feasibility to yield a wide array of analogues to facilitate the structure−activity relationship (SAR) studies. Recent research has shown some non-transition-state analogues with the ability to inhibit NAs and even drug resistant NAs, though not very effectively or selectively.[22−25] The recent reports focus on increasing the binding affinity by the development of mechanism-based covalent inhibitors, which are attacked by the nucleophilic catalytic residue Tyr406.[26,27]

As reported here, we performed high throughput screening against H1N1 and H5N1 NAs using our compound library.[28] By screening ~6800 compounds at 100 $\mu$M we were able to obtain one hit (**KR-72039**) (structure shown in Table 1), which inhibited half of the H1N1 and H5N1 NA activity ($IC_{50}$) at 37.1 ± 2.3 and 49.5 ± 2.2 $\mu$M, respectively. Compared to the known transition-state analogues (e.g., oseltamivir), which has complex synthetic routes,[29−31] this scaffold can be easily synthesized and modified as described in Scheme 1.

The pyrazolone core was synthesized by the condensation of substituted hydrazines with various $\beta$-ketoesters. The substituted aldehydes were synthesized by Meerwein arylation of substituted diazonium salts with furfural.[32] Finally,

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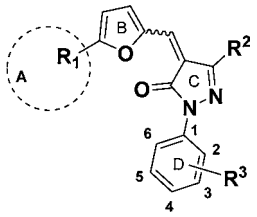
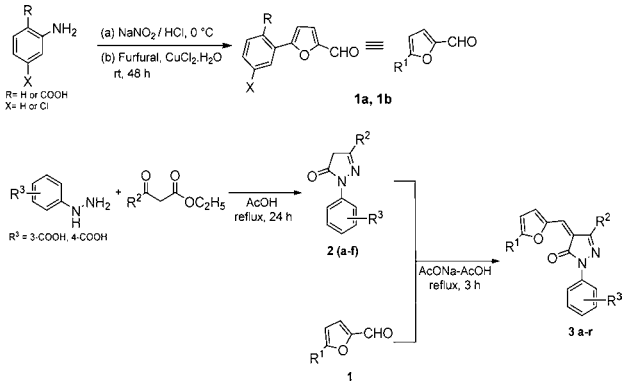
**Table 1. Results of Neuraminidase Inhibition Assay**




| number | R1 | R2 | R3 | H1N1 $IC_{50}$ ($\mu$M) | H5N1 $IC_{50}$ ($\mu$M) | H7N9-Anhui $IC_{50}$ ($\mu$M) | H7N9-Shanghai $IC_{50}$ ($\mu$M) |
|---|---|---|---|---|---|---|---|
| **KR-72039** | 2-COOH, 5-Cl-Ph | $CH_3$ | 3-COOH | 37.1 ± 2.3 | 49.5 ± 2.2 | >50 | 107.5 ± 1.6 |
| **3a** | 2-COOH, 5-Cl-Ph | $CF_3$ | 3-COOH | >50 | 3.3 ± 0.2 | >50 | >200 |
| **3b** | 2-COOH, 5-Cl-Ph | Ph | 3-COOH | >50 | 37.1 ± 0.3 | >50 | >200 |
| **3c** | H | Ph | 3-COOH | >50 | >50 | >50 | >200 |
| **3d** | H | $CH_3$ | 3-COOH | >50 | >50 | >50 | >200 |
| **3e** | 2-COOH, 5-Cl-Ph | Ph | H | >50 | 2.8 ± 0.1 | >50 | >200 |
| **3f** | 2-COOH, 5-Cl-Ph | Ph | 4-F | 32.4 ± 3.8 | 2.9 ± 0.1 | >50 | >200 |
| **3g** | 2-COOH, -Ph | $CH_3$ | 3-COOH | 44.6 ± 4.3 | 54.94 ± 7.2 | >50 | >200 |
| **3h** | 2-COOH, -Ph | Ph | H | 28.8 ± 1.6 | 13.7 ± 1.2 | >50 | >200 |
| **3i** | 2-COOH, -Ph | $CF_3$ | 3-COOH | >50 | 3.9 ± 0.1 | >50 | 109.1 ± 3.7 |
| **3j** | Ph | $CH_3$ | 3-COOH | >50 | >50 | >50 | >200 |
| **3k** | Ph | Ph | 3-COOH | >50 | >50 | >50 | >200 |
| **3l** | 2-COOH, -Ph | Ph | 4-F | >50 | 10.8 ± 0.1 | 28.1 ± 3.7 | 89.6 ± 5.9 |
| **3m** | 2-COOH, 5-Cl-Ph | $CH_3$ | H | >50 | >50 | >50 | 114.7 ± 2.7 |
| **3n** | Ph | Ph | H | >50 | >50 | >50 | >200 |
| **3o** | 2-COOH, 5-Cl-Ph | Ph | 4-$CH(CH_3)_2$ | >50 | >50 | >50 | >200 |
| **3p** | 2-COOH, -Ph | Ph | 4-CN | >50 | >50 | >50 | >200 |
| **3q** | 2-COOH, -Ph | Ph | 4-$OCH_3$ | >50 | >50 | 34.5 ± 1.9 | 108.6 ± 8.1 |
| **3r** | 3,5-COOH | $CH_3$ | 3-COOH | >50 | >50 | 42.7 ± 5.5 | 63.1 ± 0.9 |

Knoevenagel condensation of substituted aldehydes with various pyrazolones in the presence of a catalytic amount of sodium acetate in acetic acid yielded the final compounds (50−88%). The methanolic wash yielded pure compounds in most cases, thus avoiding the need for chromatographic purification.

**Scheme 1. Synthesis of Substituted Pyrazolones**




The pyrazolones with phenyl as $R^2$ were obtained as a single E-isomer, as there was no NOE signal observed between the olefinic proton and the $R^2$ phenyl protons in NMR (Figures S5−S8). However, the compounds were obtained as geometrical isomers ($E/Z$) when $R^2$ was methyl, which were inseparable due to the rapid interconversion in solution. Such isomerization has also been reported on a similar system that inhibited heptosyltransferases, such as WaaC, of Gram-negative bacteria.[33] The compounds with methyl substitution were determined to have a higher ratio of the Z isomer ($Z/E$ ratio 3:1) based on the NOE observed between the methyl of pyrazolone (2.53 ppm) and olefinic proton (7.69 ppm) in NMR (Figure S1). Therefore, the $IC_{50}$ in Table 1 for those compounds should be theoretically 4-fold smaller, if the E-isomer is contributing to the inhibitory activity.

For the ease of understanding, we have divided the molecules under study into rings A, B, C, and D (Table 1). In the case of H5N1 inhibition, replacement of the methyl substituent at $R^2$ by a phenyl ring (**3b**) resulted in a marginal increase of inhibitory activity ($IC_{50}$ = 37.1 $\mu$M). Drastic improvement was observed ($IC_{50}$ = 3.3 $\mu$M) on isosteric replacement of the methyl substitution at $R^2$ by trifluoromethyl (**3a**).

The removal of ring A was not tolerated as demonstrated by the loss of activity in the case of **3c** and **3d** irrespective of $R^2$ substitution. The loss of activity could be due to the absence of a carboxylate on ring A. The removal of carboxylate from ring A also resulted in the loss of activity as seen with **3j**, **3k**, and **3n**. Similar results were obtained when **KR-72039** was esterified (data not shown). This demonstrated that the carboxylate on ring A is a critical component.

Interestingly, while elimination of the carboxylate from ring D abolished the activity of **3m** ($R^2$ = methyl), a 15-fold increase in the potency ($IC_{50}$ = 2.8 $\mu$M) was observed for the molecule with a phenyl substituent as $R^2$ (**3e**). In fact, **3e** is the most potent inhibitor of H5N1 NA in the series. Apparently, the phenyl group as $R^2$ seems to increase the binding affinity with H5N1 NA. It is also worth noting here that while the original hit (**KR-72039**) was not selective between NAs from H1N1 and H5N1, the best compound **3e**,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along with **3a**, **3i**, and **3l**, selectively inhibited the H5N1 enzyme at a low micromolar range.

With an attempt to reduce the molecular weight, we eliminated chlorine from the ring A. This caused about a 4-fold drop in the activity when $R^2$ was a phenyl group (comparing **3e**, **3f** with **3h**, **3l** respectively). In contrast, there was no significant drop in the activity when **3a** was converted to **3i** ($R^2$ = $CF_3$).

We further determined the mode of inhibition of our best inhibitor, namely **3e**, using a Lineweaver–Burk plot (Figure 1A). This compound exhibited a mixed-mode inhibition on

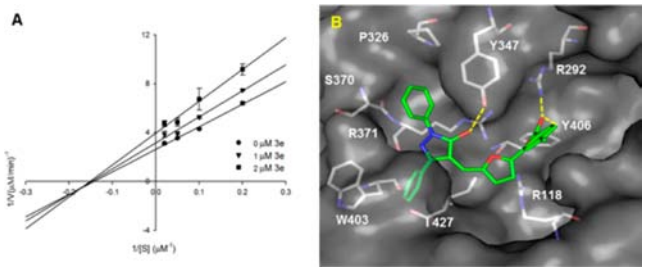


**Figure 1.** (A) Lineweaver–Burk plot to confirm mixed-type inhibition of NA hydrolase activity by **3e**. (B) Docking analysis of **3e** (PDB ID: 2HTY).

H5N1 NA with a 2-fold higher affinity to free enzyme ($K_{ic}$ = 2.9 $\mu$M) than to the enzyme–substrate complex ($K_{iu}$ = 5.6 $\mu$M) (Table S1).

We then performed molecular docking simulations to understand the possible binding interactions of our compounds with H5N1 NA. Since the enzyme kinetics experiment showed that the compound **3e** has higher affinity toward free enzyme ($K_{ic}$ = 2.9 $\mu$M), we used an apo-form NA crystal structure (PDB ID: 2HTY) for our docking analysis. The docking simulation option (Accurate docking) of iGemdock v2.1 was used to generate 20 solutions. The inhibitors with $R^2$ as the phenyl group prefer to occupy loop-430, an allosteric site of NA, and extend partly into the active site to interact with the residues therein. This is consistent with the mixed-type inhibition pattern determined experimentally. For example, in **3e** as shown in Figure 1B, $R^2$ phenyl mainly interacts with the hydrophobic side chain of I427, P431, and K432 along with the additional weak $\pi$–$\pi$ interaction with W403 in loop-430. The carboxylate moiety on ring A forms a H-bond with R292 at the catalytic site and appears to be necessary for activity. When this interaction was lost, as seen with **3h** and **3l** that interact with R156, there was about a 30% drop in activity (Figure S2). The chlorine on the ring A can help to keep the ring in a proper orientation. Additionally, there are some other reports that corroborate the binding of a noncompetitive inhibitor around the NA active site.[34−36]

A cell-based assay using live H5N1 virus showed **3e**, **3f**, and **3h** effectively inhibited the virus with an $EC_{50}$ of 27, 32, and 24 $\mu$M, respectively. This demonstrated NA as the genuine target of our compounds. All effective inhibitors were nontoxic toward HEK-293 cells at 100 $\mu$M, and their $CC_{50}$ were larger than 200 $\mu$M except in the case of **3f** (Figures S3 and S4).

We further tested our compounds against the NA of the recently emerged H7N9 virus. While elimination of carboxylate from ring A of **KR-72039** abolished the activity, its elimination from ring D (**3m**) either retained or improved the potency specifically against R292K mutant. Two compounds with $R^2$ phenyl substitution (**3l**, **3q**) and the one with $R^2$ methyl substitution (**3r**) inhibited both the wild-type and the mutant H7N9 NA.

Interestingly, **3l** was a common inhibitor for both H5N1 and H7N9 NAs. It is also worthwhile to note that **3l** inhibits H7N9-R292K mutant NA from H7N9-Shanghai with an $IC_{50}$ of 90 $\mu$M, merely 3-fold higher than that in inhibiting wild-type H7N9 NA from H7N9-Anhui. In contrast, oseltamivir inhibits H7N9-R292K mutant NA with an $IC_{50}$ of 9 $\mu$M but with a >1000-fold difference between inhibiting the wild-type and the mutant H7N9 NAs.[37] To the best of our knowledge, this is the first nontransition-state analog reported to inhibit H7N9 NAs. We are continuing our effort to understand exact binding of the molecule in N1 and N2 NAs by X-ray crystallography. This will provide us with a better understanding of the binding modes in these NAs and facilitate further modification.

In summary, this study resulted in the discovery of a pyrazolone scaffold with dual advantages: a simple synthetic approach and larger molecular diversity. Modifications led to **3a**, **3b**, and **3e**, selective low micromolar inhibitors of H5N1 NA, with **3e** as the most potent one *in vitro* and *in vivo*. Moreover, we identified **3l**, a dual inhibitor of H5N1 and recently emerged H7N9 NAs. Since our compounds have been verified for *in vitro* and *in vivo* efficacy and are devoid of toxicity at doses as high as 200 $\mu$M, they could act as suitable leads for anti-AI virus drug development.

## ■ ASSOCIATED CONTENT

### Supporting Information

Synthesis details, $^1$H and $^{13}$C NMR of synthesized compounds, and assay methods. This material is available free of charge via the Internet at http://pubs.acs.org.

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### Notes

The authors declare no competing financial interest.

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