Original Article

A microplate-based screening assay for neuraminidase inhibitors

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ABSTRACT: Neuraminidase (NA) represents a highly promising new target for drug development in influenza virus genes. Rapid screening of enzyme inhibitors is a key method for the identification of leading compounds. In order to speed up the screening for enzyme inhibitors of natural and synthetic origin, effective and fast assays are needed. 2'-(4-Methylumbelliferyl)-α-D-*N*-acetylneuraminic acid (4-MUNANA) was selected as substrate for development of a microplate-based assay. The enzymatic reaction conditions were optimized as follows: in a 100 μL reaction mixture, the final concentrations were 32.5 mM sodium acetate (pH 3.5), 20 μM 4-MUNANA, 0.005% (w/v) bovine serum albumin, and 0.42 μg/mL NA. In the study, the dose-response relationship of oseltamivir carboxylate to NA activity was observed. In addition, an overall Z' value of 0.8 proved the systems robustness and potential for screening. The assay system developed will be a valuable tool to discover new structures for the therapeutic inhibition of NA used to treat Influenza.

Keywords: Neuraminidase, 4-MUNANA, microplate-based, screening assays, NA inhibitors

1. Introduction

Influenza is an acute viral infection of the upper respiratory tract that can affect millions of people every year. It is a high-priority and attractive area for drug discovery to develop effective anti-influenza agents. The influenza virus surface glycoprotein antigen neuraminidase (NA) has been proven as a valid therapeutic target for antiviral drugs due to its essential role in the viral replication cycle (*1,2*). NA catalyzes removal of terminal sialic acid linked to glycoproteins and glycolipids, which promotes influenza virus release from infected cells and facilitates virus spread within the respiratory tract. Despite the homologous identity of NA in different strains being only about 30%, the catalytic site of NA in all influenza A and B viruses is completely conserved (*3*). The enzymatic active site of NA contains four anti-parallel strands arranged in a propeller fashion. Each monomeric subunit has an active site cavity lined with 10 conserved residues and four water molecules. The inhibitors bind to the active site with the carboxylic acid binding to the triad guanidine groups of the three arginine residues, Arg 118, Arg 371, and Arg 292, which are located as a cluster on one side of the active site. Opposite to the guanidine triad, there is a hydrophobic pocket formed by side chains of Trp178, Ile 222, and part of Arg 224 (*4*) (Figure 1).

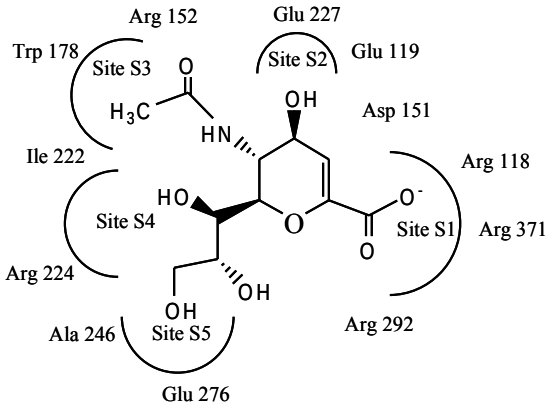

Figure 1. 'Airplane' model of NA active site (*5*)**.**

Therefore, NA has been regarded as an attractive target for antiviral drug development. So far, two NA inhibitors (zanamivir and oseltamivir) have been confirmed as effective and safe for the treatment of influenza and have been approved by FDA (*6*).

Rapid screening of enzyme inhibitors is a key method for the discovery of leading compounds. In order to speed up screening for enzyme inhibitors of natural and synthetic origin, effective and fast assays are necessary. The choice of an appropriate screening assay is crucial. Screening assays for enzyme inhibitors consist of *in vivo* and *in vitro* assay methods. *In vitro* screening assays include cell-based and biochemically-based

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methods. In biochemically-based assays, the target is specific, and the drug action mechanism can be obtained directly. They allow for efficient logistics, and rapid screening of large libraries can be easily miniaturized, without suffering from off-target effects and toxicity issues. Biochemically-based assays can be performed with higher compound concentrations, thereby raising chances of finding inhibitors in novel chemical classes (*7*). Microplate-based assays are one form of biochemically-based assays, which have undergone a revolution and the field shows no signs of slowing down. This has been made possible through the introduction of high density microplates, small volume liquid handling robotics and associated detection technology. The common methods mainly use homogeneous formats that do not involve any solid phases or washing steps. The development of homogeneous mix-and-measure techniques was a necessary precondition for the current level of miniaturization and throughput in screening laboratories.

It was used to screen NA inhibitors using biochemically-based assays. Hong-Peng Cao and his partners have reported screening NA inhibitors using 4-MUNANA as substrate (*8*). NA was prepared from strain A (Yuefang 72-243 A and Jifang 90-15 A) and B (Sichuan 2000-38 B) influenza viruses. NA from different species has different reaction conditions. In this paper, we also chose 4-MUNANA as substrate to develop a microplate-based assay for neuraminidase inhibitors. NA was prepared from *Clostridium perfringens*, which has a high reaction activity. The reaction conditions were optimized. NA activity was monitored by using a synthetic substrate 4-MUNANA, which is hydrolyzed to yield a fluorescent product 4-methylumbelliferone (4-MU) that can be quantified using a fluorometric method (*9*). The intensity of fluorescence reflects the activity of NA sensitively.

2. Materials and Methods

2.1. *Enzyme and reagents*

NA, 4-MUNANA, and 4-MU were purchased from Sigma Chemicals (St. Louis, MO, USA). Black fluorescence 96-well plates were purchased from SPL chemical (South Korea). Tamiflu® capsules were obtained from F. Hoffmann, La Roche Ltd. (Basel, Switzerland, the labeled amount of 75 mg oseltamivir corresponded to 98.5 mg oseltamivir phosphate). The 20 chemical compounds were synthesized by Jianzhi Gong. All other chemicals were analytical-grade commercial preparations.

2.2. *Preparation of oseltamivir carboxylate*

Oseltamivir (Tamiflu®) is rapidly and extensively hydrolysed *in vivo* to its active metabolite oseltamivir carboxylate, which is a potent and selective inhibitor of influenza virus NA.

We transfered an accurately weighed portion of the contents of 5 opened Tamiflu® capsules, equivalent to about 25 mg of oseltamivir, to a 25-mL volumetric flask, added about 15 mL of water, and shook by mechanical means for about 15 min. We diluted with water to volume, and mixed. A portion of this solution was centrifuged, and 8.0 mL of the clear supernatant was transferred to a 20-mL round bottom flask, 2.0 mL of 10% sodium hydroxide was added, and mixed. The solution was refluxed for 3 h, cooled to room temperature, and adjusted to pH 7.5 with glacial acetic acid.

The hydrolyzed ratio of oseltamivir was determined using the method described by N. Lindegardh (*10*). The hydrolyzed ratio was calculated by comparing the peak area of oseltamivir in solution before and after hydrolyzation. We found that oseltamivir was hydrolyzed into oseltamivir carboxylate completely.

2.3. *Determination of the stability of NA*

NA (16.7 μg/mL) was prepared in redistilled water and 0.1% cold bovine serum albumin solution, respectively. The stability of NA in these solvents was monitored by determining the reaction activity of NA using optimized conditions.

2.4. *Determination of the stability of 4-MU*

4-MU (20 μM) was prepared in 0.15 M glycine-NaOH buffer (pH 10.4) and 50 mM sodium acetate buffer (pH 3.5), respectively. The intensity of fluorescence was measured at an excitation wavelength of 355 nm and an emission wavelength of 460 nm.

2.5. *Determination of neuraminidase activity*

The activity of NA was measured fluorometrically by determination of the degradation product 4-MU from the substrate 4-MUNANA. The reaction was performed under the following experimental conditions: 50 mM sodium acetate reaction buffer (pH 3.5), 0.2 mM 4-MUNANA, 0.15 M glycine-NaOH stop buffer (pH 10.4). The activity of the enzyme was measured in black fluorescence 96-well plates using the following procedure: 5 μL of NA (8.33 μg/mL) and 10 μL of 0.2 mM 4-MUNANA were added to 85 μL of 50 mM sodium acetate buffer (pH 3.5). The reaction mixture was gently stirred at 37°C for 15 min and 100 μL of stop buffer was added to the reaction solution. Meanwhile, a substrate blank control was set. Five μL of deactived NA (8.33 μg/mL) was added to the reaction mixture instead of 5 μL of NA (8.33 μg/mL). The fluorescence intensities were measured using an excitation wavelength of 355 nm and an emission wavelength of 460 nm.

2.6. *Validation of the assay*

The oseltamivir carboxylate solution was diluted into six concentrations with redistilled water: 783.9, 156.8, 31.36, 6.27, 1.25, and 0.25 μg/mL. Five μL of NA (8.33 μg/mL) was preincubated with 10 μL of oseltamivir carboxylate in 50 mM sodium acetate buffer (pH 3.5) at 37°C for 30 min, respectively. Ten μL of 0.2 mM 4-MUNANA was then added to each well. The terminal volume was 100 μL. The reaction was stopped after 15 min incubation by adding 100 μL of stop buffer. Meanwhile, substrate blank control (deactived NA) and enzyme activity control (no inhibitors) were set. The fluorescence intensities were measured using the method described above in order to obtain the inhibition ratio of oseltamivir carboxylate to NA activity.

$$\textbf{Inhibition ratio} = \frac{\mathbf{F}_{\text{enzyme activity control}} - \mathbf{F}_{\text{test}}}{\mathbf{F}_{\text{enzyme activity control}} - \mathbf{F}_{\text{black control}}} \times 100\%$$

2.7. *Inhibitor screening assays*

A total of 20 chemical compounds were used for screening and were dissolved in 50 mM sodium acetate buffer (pH 3.5) to a concentration of 2 mg/mL. Five μL of NA (8.33 μg/mL) was preincubated with 20 μL of various compounds (2 mg/mL) in 65 μL sodium acetate buffer (50 mM, pH 3.5) at 37°C for 30 min. Ten μL of substrate 4-MUNANA (0.2 mM) was then added to each well. The reaction was stopped after 15 min incubation by adding 100 μL of stop buffer. Meanwhile, substrate blank control (deactived NA) and enzyme activity control (no inhibitors) were set. The fluorescence intensities were measured using the method described above.

3. Results and Discussion

3.1. *Assay conditions and optimization*

Since the aim of this assay is the identification of small molecules that bind and inhibit the screened target, optimization translates into maximizing sensitivity towards inhibitors while maintaining good statistical quality and keeping reagent costs low.

3.1.1. *Stability of NA in solutions*

The activity of NA in solutions was not only retained but also increased as described according to the instructions. We, therefore, needed to investigate the stability of NA in different solvents and at different times to ensure that the NA solution was stable when used. When the enzyme was dissolved at a concentration of 16.7 μg/mL in redistilled water, it not only retained activity at 4°C but, actually increased activity in 10 days, and was comparatively stable from 11 to 15 days. When dissolved at 16.7 μg/mL in 0.1% cold bovine serum albumin solution, the activity of NA increased about 7 folds compared to activity in redistilled water. It was stable for 14 days before it increased in activity.

From the results, NA in 0.1% bovine serum albumin solution was stable for a much longer time. Meanwhile, the activity of NA increased compared to that in redistilled water. So, NA (8.33 μg/mL) prepared in 0.1% cold bovine serum albumin solution for 14 days was used.

3.1.2. *Stability of 4-MU in solution*

In 0.15 M glycine-NaOH buffer (pH 10.4), the fluorescence of 4-MU was constant for at least 10 h. In 50 mM sodium acetate buffer (pH 3.5), fluorescence was constant for at least 30 min. Therefore, 4-MU was stable in the reaction mixture, which meets the requirements for determination.

3.1.3. *Substrate concentrations*

In order to obtain the best reaction conditions, we determined the effect of substrate concentrations on NA activity. Various concentrations of 4-MUNANA (3, 6, 9, 18, 27, 36, and 54 μM) were incubated with the NA at 37°C for 5, 10, and 15 min, respectively. With the increase of substrate concentration the fluorescence intensity increased gradually (Figure 3). The enzymatic reaction rate was constant at 15 min when substrate concentration was 9, 18, 27, 36, and 54 μM. When substrate concentration was 9, 18, 27, and 36 μM, the

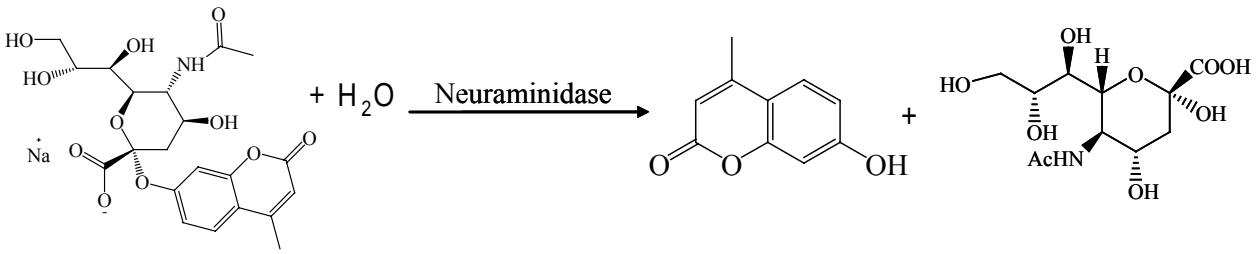

Figure 2. Reaction principle of microplate-based screening assay for NA inhibitors. NANA: *N*-Acetylneuraminic Acid.

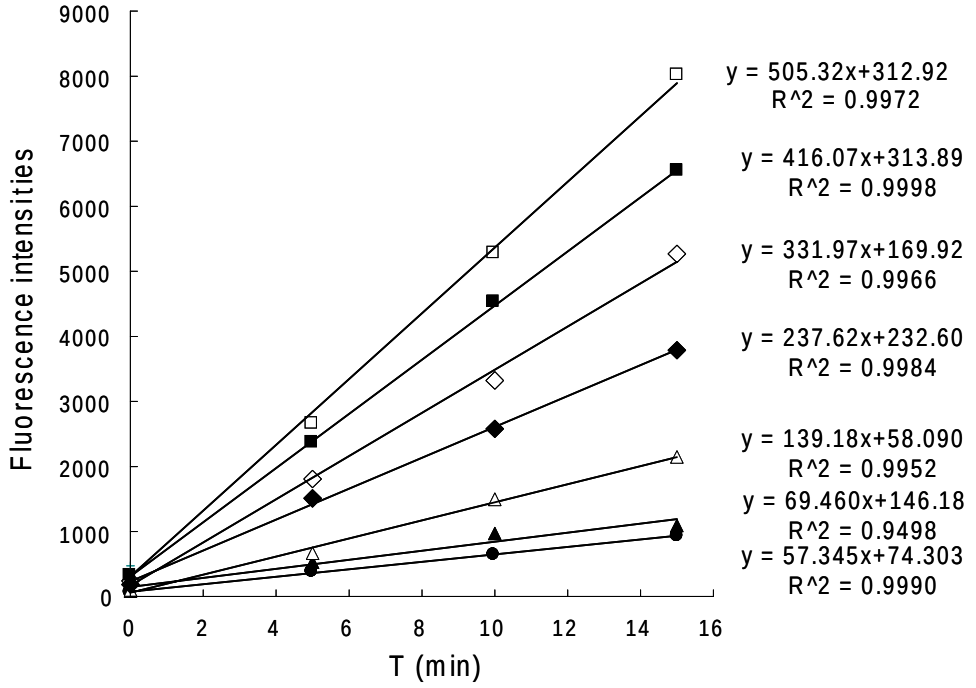

Figure 3. Effect of substrate concentrations on the NA activity. Substrate concentrations: 3 μM (●); 6 μM (▲); 9 μM (Δ); 18 μM (◆); 27 μM (◊); 36 μM (■); 54 μM (□).

values of B/S were smaller, and substrate fluorescence did not markedly interfere with the determination, which increased the sensitivity of detection (Figure 4). In order to reduce the interference and the cost, we chose 20 μM for the substrate concentration.

$$B/S = F_s / F_m \times 100\%$$

F_s and F_m are the fluorescence intensities measured after a 15 min incubation with deactivated and active enzyme.

3.1.4. *The pH of sodium acetate buffer*

Fifty mM sodium acetate buffer at various pHs (pH 3.0, 3.5, 4.0, 4.5, 5.0, 6.0, and 7.0) was used as reaction buffer to determine the activity of NA. The activity of NA increased from pH 3.0 to pH 3.5, and decreased when pH was changed from 3.5 to 7.0 (Figure 5). We chose 50 mM sodium acetate buffer (pH 3.5) as reaction buffer.

3.1.5. *Calcium ions*

Various concentrations of $CaCl_2$ prepared in 50 mM sodium acetate buffer (pH 3.5) were used to determine the activity of NA by the method described above. The reaction characteristics of NA vary with biological source. Only neuraminidase from Vibrio cholera requires calcium ions. However, the presence of calcium ions does not interfere with the activity of neuraminidases from other sources. In this study, NA

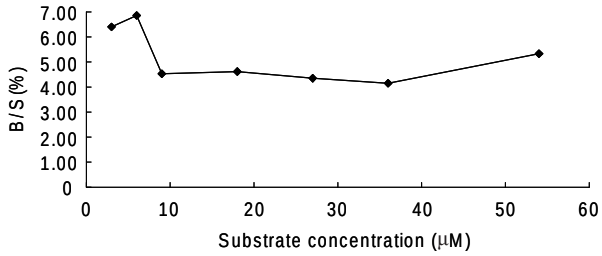

Figure 4. The interference of substrate concentrations on the determination.

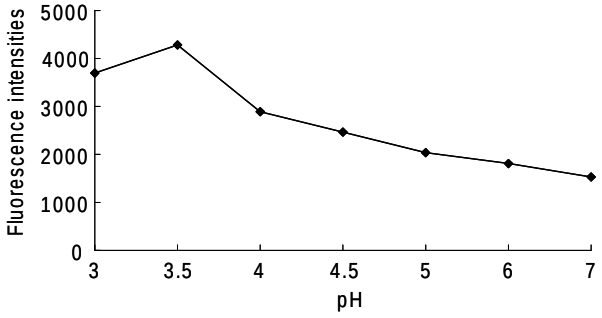

Figure 5. Effect of the pH of sodium acetate buffer on the determination of NA activity.

was prepared from *Clostridium perfringens*. The results demonstrated that NA from *Clostridium perfringens* does not require calcium ions for activity (Figure 6). This was in reasonable agreement with the published literature (*11*).

3.1.6. *Temperature*

The activity of NA was determined at various temperatures (25, 37, 40, 50, 60°C). The results demonstrated that the optimal temperature was 37°C.

3.1.7. *K_m value of the NA for 4-MUNANA*

K_m value of the NA for 4-MUNANA was determined by initial-rate enzyme assay. In order to ensure the progress of the reaction followed Michaelis-Menten kinetics, we chose 10 min as reaction time. The K_m value was calculated as 46.97 μM from a double-reciprocal plot (Figure 7).

3.2. *Assay validation*

We first investigated the assay's ability to identify known inhibitors. The IC_{50} value of previously characterized NA inhibitors was determined employing this assay by taking a fluorescent measurement after a reaction time of 15 min. Oseltamivir carboxylate was used as a known inhibitor for NA. The dose-response relationship of oseltamivir carboxylate to NA activity was observed. The IC_{50} value of oseltamivir carboxylate was 2.29 μM, which was in reasonable agreement with the published literature (*4*), thereby confirming its feasibility for screening.

Secondly, to quantify assay performance, we calculated the Z' factor, which was a main quality parameter in HTS, as described by Equation (1), where σ is the standard deviation and μ is the mean of the standard (s) or the negative (b) control (100% inhibition by a reference inhibitor) (*12*).

$$Z' = 1 - \frac{3\sigma_s + 3\sigma_b}{|\mu_s - \mu_b|} \qquad (1)$$

A Z' factor above 0.5 indicates a large separation band between the values for the positive and negative controls (100% and 0% activities, respectively). The Z' factor has the advantage of expressing the noise in relation to the signal window and, thus, gives a more complete estimation of assay quality than signal-to-background or signal-to-noise ratios alone would do (*7*). In this case, values between 0.8 and 0.9 were found, demonstrating that the assay holds a large separation band between samples and blank signals and thereby confirming its sensitivity for screening.

All of these findings establish the reliability and reproducibility of the developed fluorescent screening system for NA inhibition.

3.3. *Inhibitor screening assays*

Using the assay described above, 20 chemical compounds were used for screening. All the compounds were small molecule pyrrolidine derivatives, which were potencial NA inhibitors. In the primary screening, we found six compounds were active and the inhibition ratio was above 20%. For secondary screening, each of the six compounds was prepared in five concentrations. In our study, we observed a dose-response relationship of compound 10m for NA activity with an IC_{50} value of 2.96 mM (Figure 8). From the result, compound 10m (IC_{50} = 2.96 mM) was less effective against NA than oseltamivir carboxylate (IC_{50} = 2.29 μM), and will probably not be a leading compound. The synthesis and screening was continued in order to find leading compounds against NA.

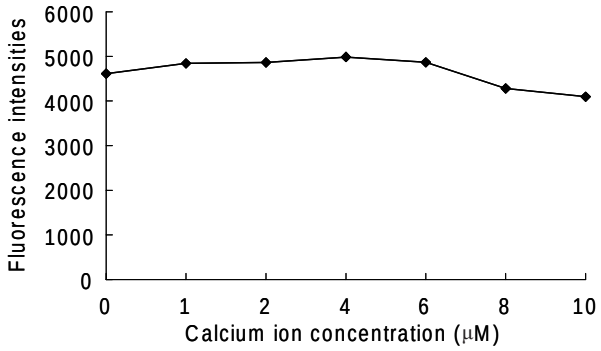

Figure 6. Effect of calcium ion on NA activity.

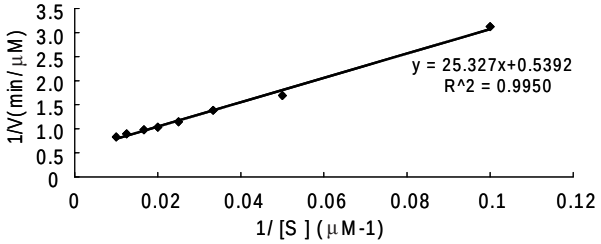

Figure 7. Assessment of K_m value of the NA for 4-MUNANA.

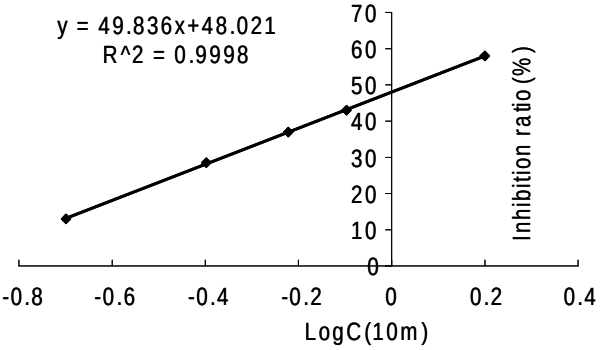

Figure 8. The inhibition curve of 10m to NA activity.

4. Conclusion

In the current study, we have developed and validated a fluorescent assay for the HTS of NA inhibition. This

test system will facilitate the HTS of large compound libraries with the objective to discover currently unknown potent NA inhibitors leading to novel drugs for influenza treatment. One shortage of this assay is that it is not suitable for screening mixtures. Further work will focus on screening for compounds synthesized by combinatorial chemistry. These results will be reported in future papers.

Acknowledgements

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