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RESEARCH PAPER

A microfluidics-based mobility shift assay to identify new inhibitors of β-secretase for Alzheimer's disease

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Abstract The β-secretase (BACE1) initiates the generation of toxic amyloid-β peptide (Aβ) from amyloid-β precursor protein (APP), which was widely considered to play a key role in the pathogenesis of Alzheimer's disease (AD). Here, a novel microfluidics-based mobility shift assay (MMSA) was developed, validated, and applied for the screening of BACE1 inhibitors for AD. First, the BACE1 activity assay was established with a new fluorescent peptide substrate (FAM-EVNLDAEF) derived from the Swedish mutant APP, and high-quality ratiometric data were generated in both endpoint and kinetic modes by electrophoretic separation of peptide substrate from the BACE1 cleaved product (FAM-EVNL) before fluorescence quantification. To validate the assay, the inhibition and kinetic parameter values of two known inhibitors (AZD3839 and AZD3293) were evaluated, and the results were in good agreement with those reported by other methods. Finally, the assay was applied to screen for new inhibitors from a 900-compound library in a 384-well format, and one novel hit (IC_{50} = 26.5 ± 1.5 μM) was identified. Compared with the common fluorescence-based assays, the primary advantage of the direct MMSA was to discover novel BACE1 inhibitors with lower auto-fluorescence interference, and its superb capability for kinetic study.

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Introduction

Alzheimer's disease (AD) is a degenerative disease characterized by severe memory loss and neuronal cell death. Today, 47 million people suffer from dementia worldwide, with AD as the most common cause accounting for an estimated 60–80% of these cases [1, 2]. The aggregation of 40- or 42-amino-acid-long amyloid-β peptide ($Aβ_{40,42}$) in human brain is regarded as one main reason for the pathogenesis of Alzheimer's disease [3]. $Aβ_{40,42}$ is generated after sequential cleavage of the β-amyloid precursor protein (APP) via β- and γ-secretase, where β-secretase (BACE1) acts as the rate-limiting enzyme [4–6]. Given that BACE1 is the initiating and rate-limiting enzyme in Aβ generation, it is considered as the prime target for AD therapy [7].

Since the statine-based inhibitors were identified in 2000, many BACE1 inhibitors with different structures have been designed and developed [8]. Four of them have entered into phase III clinical trials, including AZD3293, CNP520, JNJ-54861911, and MK-8931 [9]. Since none of them has been approved by FDA to date, additional potent candidates especially those with different structures and inhibition mechanisms are urgently needed.

Various methods have been developed for the screening of BACE1 inhibitors or ligands, which have been summarized by Mancini et al. [10], including classic fluorescence resonance energy transfer (FRET) [11–13], homogenous time-resolved fluorescence (HTRF) [14], fluorescence polarization [15], liquid chromatography in combination with an immobilized enzyme reactor [16, 17], capillary electrophoresis-mass spectrometry (CE-MS) [18], surface

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plasmon resonance [19], AlphaScreen [20], and isothermal titration calorimetry [21]. Among them, the FRET-based methods are commonly used because they are homogenous and sensitive assays with high throughput capabilities. However, there are several drawbacks that need to be considered, such as low assay sensitivity for the contribution of the uncleaved fluorophores to the background signal and interferences arising from the absorbing or fluorescent compounds. An improved HTRF assay was developed by Kennedy et al. [14]. The assay allows for a time delay between excitation of donor and readout of acceptor-emission, which virtually eliminates any intrinsic short-lived fluorescence. But the HTRF assay cannot be used in a continuous manner, which limits its application in kinetic studies. Thus, an unambiguous and continuous in vitro assay is required to reduce the autofluorescence interference and characterize the mechanisms of inhibitors.

The microfluidics-based mobility shift assay (MMSA) involves the on-chip electrophoretic separation and quantification of the fluorescence-labeled substrate and product based on the changes in charge and/or mass [22]. Thus, an important advantage of the assay is that the potential interference of fluorescent compounds will be minimized and monitored because of the direct readout of substrate conversion. Meanwhile, the ability of monitoring enzymatic reactions in real time facilitates the kinetic study. Therefore, the MMSA technology has been used in a wide type of targets, including kinases, proteases, phosphatases, histone deacetylases, phosphodiesterases, acyl-transferases, and non-enzymatic small molecule binders of DNA/RNA [23]. In this study, a new microfluidics-based mobility shift assay was developed, validated, and applied for the screening of BACE1 inhibitors in both kinetic and endpoint modes.

Materials and methods

Reagents

Recombinant BACE1 (amino acids 22–457) was obtained from Abcam (Cambridge, USA). The fluorescence-labeled APP Swedish (FAM-APPsw) peptide substrate, FAM-EVNLDAEF, designed in the MMSA was synthesized by GL Biochem (Shanghai, China). Compounds AZD3839, AZD3293, and donepezil were obtained from Selleckchem (Houston, USA). The Maybridge HitFinder™ library was from Fisher Scientific (Loughborough, UK). Black standard plates (384-well) were from Corning (New York, USA). G-50 Sephadex spin columns were purchased from Roche (Basel, Switzerland). All other reagents were purchased from Sigma-Aldrich (St. Louis, USA). Water was purified using a Millipore apparatus (Billerica, USA).

Instrumentation

The MMSA was carried out on a LabChip EZ Reader II instrument from PerkinElmer (Waltham, USA). Nanoliter samples from each well were sipped by a 12-sipper chip where electrophoretic separation of substrate (FAM-EVNLDAEF) and product (FAM-EVNL) occurred under a screen pressure of − 1.5 psi, an upstream voltage of − 300 V, and a downstream voltage of − 1400 V. The size of 12-sipper chip (in the glass, not including the plastic caddy) is 57 × 57 mm, channel width is 25 μm and depth is 10 μm, and effective separation length is about 29 mm. After a 29-mm-long separation in each channel, the fluorescent analytes pass through an optical window where they were simultaneously stimulated by a blue LED (450–490 nm) and detected by a CCD camera (515–550 nm). The separation buffer contained 100 mM HEPES, 1 mM EDTA, 0.015% Brij-35, 0.2% coating-3 reagent (PerkinElmer, Waltham, USA), and 5% DMSO, pH 7.5. The substrate conversion (SC) was defined as the peak height of product divided by the sum of both substrate and product.

Inhibition studies of enzymatic reaction

Assays were performed in reaction buffer (50 mM sodium acetate, pH 4.5, 0.1% Brij-35, and 10% glycerol) in a 384-well format. BACE1 solution (15 μL, 1 nM final enzyme concentration in reaction buffer) was added to 250 nL compound solution (dissolved in 100% DMSO with various concentrations) or 250 nL DMSO as controls pre-plated in assay plate by Echo550 (Labcyte, San Jose, USA). For positive control wells, 15 μL reaction buffer alone was added instead of BACE1 solution. After the enzyme and inhibitor were pre-incubated at 25 °C for 1 h, reactions were started by addition of 10 μL FAM-APPsw peptide (5 μM final concentration). After 1-h incubation at 25°C, the reactions were terminated by adding 25 μL stop buffer (200 mM HEPES, pH 7.5, 2 mM EDTA, 0.03% Brij-35, 0.4% coating-3 reagent, 2 μM AZD3839). The percentage of inhibition was calculated as $\frac{\left(SC_{sample}-SC_{negative}\right)}{\left(SC_{positive}-SC_{negative}\right)}$. To generate the IC_{50} values, the dose–response curves of BACE1 inhibitors were fitted to Eq. (1):

$$Y = Bottom + \frac{(\mathrm{Top}-Bottom)}{\left(1+10^{((\mathrm{LogIC}_{50}-X)\times Hillslope)}\right)}$$

where Y is the percentage of inhibition, Top and Bottom represent maximum and minimum values of Y, respectively, and X = log (molar compound concentration).

Kinetic studies of enzymatic reaction

To characterize the kinetic parameters of enzyme, BACE1 (1 nM final concentration) and FAM-APPsw peptide at 15 to

250 μM were incubated at 25 °C for 1 h. The calculated product formation rates and substrate concentrations were fitted to a Michaelis–Menten Eq. (2) to obtain the K_M and V_{max} values:

$$Y = V_{max} \times \frac{X}{(K_M + X)}$$

where Y is the calculated product formation rates and X is the substrate concentration.

For determination of association rate constant (k_{on}), FAM-APPsw peptide was mixed with compound at different concentrations before the reactions were initiated by the addition of BACE1 solution. The samples were then quickly measured continuously over a 2-h period. To obtain the k_{on} value for time-dependent inhibitors, the reaction progress curves at various inhibitor concentrations were fitted globally to the following equations (3–4):

$$Y = \left(c + v_s \times t + \frac{(v_i - v_s) \times (1-g)}{(k_{obs} \times g)} \times \ln\left(\frac{\left(1 - g \times e^{(-k_{obs} \times t)}\right)}{(1-g)} \right) \right)$$

$$k_{obs} = k_3 \times [I] + k_4$$

where Y is the substrate conversion, g is defined as $g = \frac{E}{[I]} \times \left(1 - \frac{E}{[I]}\right)^2$, v_i is the maximal velocity of the reaction without inhibitor, v_s is stable velocity of the reaction for each inhibitor concentration, $[I]$ is the inhibitor concentration, E is the enzyme concentration, k_{obs} is the apparent first-order rate constant for the transition from v_i to v_s, t is time, and k_3 and k_4 represent the association and dissociation rate constant of the enzyme–inhibitor complex, respectively.

For determination of dissociation rate constant (k_{off}), BACE1 solution was pre-incubated with inhibitor at 10 × IC_{50} in assay buffer at 25 °C for 1 h to allow the formation of the BACE1–inhibitor complex. The complex was then purified through a G-50 Sephadex spin column to remove the free inhibitor according to the manual. The reaction was initiated by rapidly diluting the purified enzyme–inhibitor complex into the FAM-APPsw peptide solution. Recovery of enzymatic activity was monitored continuously over a 400-min period. To obtain the k_{off} value of inhibitors, the recovery of enzymatic activity over time was fitted to Eq. (5):

$$Y = v_s \times t + \left(\frac{v_i - v_s}{k_{obs}} \right) \times \left(1 - e^{(-k_{obs} \times t)}\right)$$

where Y is the substrate conversion, v_i and v_s are the initial and steady-state velocities of the reaction in the presence of the inhibitor, k_{obs} is the apparent first-order rate constant for the transition from v_i to v_s, and t is time. Under the experimental conditions, k_{obs} approximates the dissociation rate constant (k_{off}) of the enzyme–inhibitor complex.

The enzyme–inhibitor dissociation half-life ($t_{1/2}$) is calculated through Eq. (6): $t_{1/2} = \frac{0.693}{k_{off}}$. All the above equations can be referred to the literature [24].

Pilot screening of novel inhibitors

Nine hundred compounds from Maybridge HitFinder™ library were screened for their efficacy against BACE1. Compounds were dissolved in DMSO and screened at 20 μM in duplicate with the MMSA. Further dose–response curves were performed on hits using 0.01–200 μM of compounds. To evaluate the quality of assays, Z' factor was calculated using the following equation (7):

$$Z' = 1 - 3 \times \frac{(\text{SD } of \text{ high control} + \text{SD } of \text{ low control})}{(\text{mean } of \text{ high control} - \text{mean } of \text{ low control})}$$

Homogeneous time-resolved fluorescence assay

The further validation of the above hits was performed with a BACE1 HTRF assay. It has been developed by Kennedy et al. [14], and minor modifications were made in our study. Briefly, 5 μL of BACE1 (3 nM final concentration) was pre-incubated with 100 nL compound in 100% DMSO or 100 nL DMSO as controls at 25 °C for 1 h in a 384-well white low volume plate (PerkinElmer, Waltham, USA). For positive controls, 5 μL reaction buffer (20 mM PIPES, pH 5.0, 0.1% Brij-35, and 10% glycerol) was added instead of BACE1 solution. Reactions started by the addition of 5 μL substrate mixture containing 50 nM EuK-APPsw and 10 μM unlabeled-APPsw peptides (Cisbio, Marcoules, France). After 4-h incubation at 25 °C, the reactions were terminated by adding 10 μL stop buffer containing 50 mM Tris–HCl, pH 8.0, 0.5 M KF, 0.001% Brij-35, and 20 μg/mL SA-XLent (Cisbio, Marcoules, France). The percentage of inhibition was calculated as $\frac{(R_{sample} - R_{negative})}{(R_{positive} - R_{negative})}$. R represents the ratio of the 665/615 nm × 10^4.

Results and discussion

Mobility shift assay development and optimization

As mentioned above, compound interference becomes the intrinsic drawback of common fluorescence-based assays. We were interested in developing a direct assay to minimize potential interference and also to perform kinetic studies. Toward that end, a fluorescent peptide derived from the Swedish mutant APP, FAM-EVNLDAEF, was designed as the substrate for the MMSA (Fig. 1a). High-quality ratiometric data were generated in both endpoint and kinetic modes by

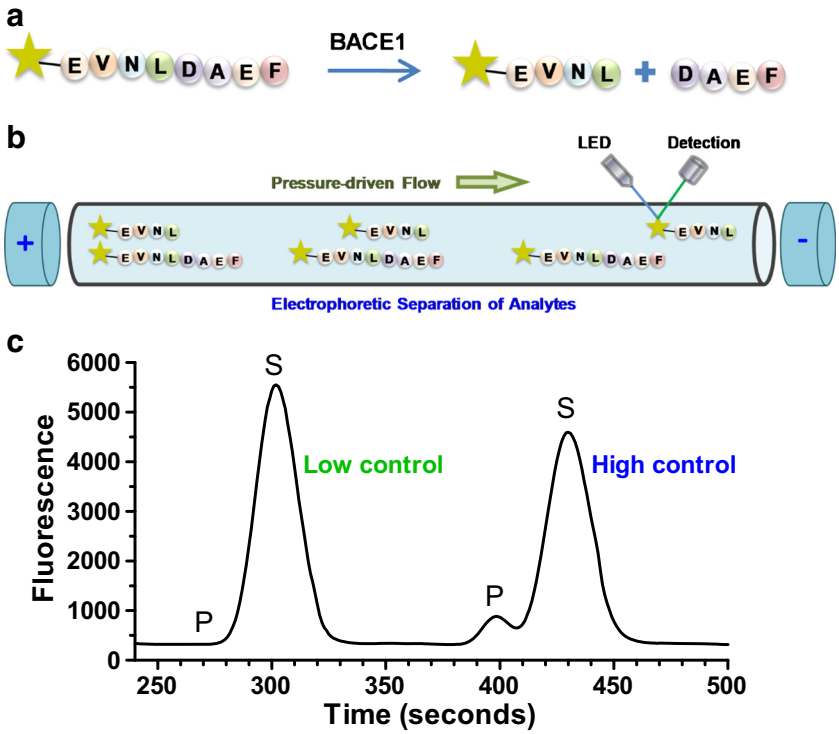

Fig. 1 Microfluidics-based mobility shift assay for BACE1. **a** The peptide substrate (FAM-EVNLDAEF) was recognized and cleaved by BACE1 to yield a product (FAM-EVNL). **b** Shift in mobility of substrate and product when separated in the chip by electrophoresis and detected via LED induced fluorescence. **c** This tracer showed a view of two wells (low control and high control, respectively) of data from EZReader II and redrawn using the GraphPad Prism 5 software. In this experiment, fluorescent product (P) moved faster through the chip and reached the detection window first. The substrate conversion (SC) was defined as the peak height of product divided by the sum of both substrate and product. The two peaks on the right from high control showed 11.8% conversion of substrate (S at 430 s) to product (P at 398 s). The peaks on the left from low control well showed 0.31% conversion (S at 302 s, P at 270 s), which reflected the low background of the assay

electrophoretic separation of peptide substrate from the BACE1 cleaved product (FAM-EVNL) (Fig. 1b). As shown in Fig. 1c, the two peaks on the right from high control well showed that the separation of the substrate (S at 430 s) and product (P at 398 s) was achieved within 2 min in MMSA (be able to get 12 data points within 2 min for 12-sipper chip), which was shorter than CE-MS (9 min) [18] but longer than FRET and HTRF assays. The low background of the assay (0.31% substrate conversion for the low control sample) should also be noted. In our previous work, we found that the two species could be separated thoroughly once the upstream voltage changed from − 1400 to − 1550 V and the required separation time would be longer (6 min) (see Electronic Supplementary Material (ESM) Fig. S1), but the IC_{50} values of known inhibitors did not change between the two conditions (Fig. S2 in the ESM). Subsequently, the characterization of BACE1 was studied using the newly developed peptide substrate.

When BACE1 was titrated in the range of 0.078–2.5 nM, a concentration-dependent linear change in substrate conversion was exhibited (Fig. 2a). In the time course experiment, the reaction progress curves were linear for at least 3 h when 1 nM or 0.5 nM BACE1 was used (Fig. 2b). The result showed that lower BACE1 concentration can be used in MMSA than those in most FRET assays (10–470 nM for five assays and 1 nM for one assay) [10], which offered the ability to characterize the sub-nanomolar inhibitors. Figure 2c shows a Michaelis–Menten kinetics plot of the product formation rates over substrate concentrations, and the kinetic parameters K_M and V_{max} were calculated as 141 μM and 4.97 μmol/min/mg, respectively. The K_M value of the fluorescently labeled substrate in MMSA (FAM-EVNLDAEF) was higher than that of the FRET substrate (Lys(dabsyl)-SEVNLDAEFR-Glu(Gly-PEGA)-Lucifer Yellow) (9 μM) [11] but lower than that of the CE-MS unlabeled substrate (SEVNLDAEFR) (1.8 ± 0.3 mM) [18]. The distinct structures of substrates might account for the different affinities of these APP-derived peptides toward BACE1 within the three assays. It should be noted that all the above substrates were composed of the eight residues (EVNLDAEF), but CE-MS and FRET substrates were actually two amino acid residues longer than the substrate used in MMSA (one additional residue on the both the N and C terminus).

In terms of robustness, the MMSA was excellent with the Z' factor value calculated as 0.82 from 20 positive controls and 20 negative controls (Fig. 2d). Furthermore, the assay window

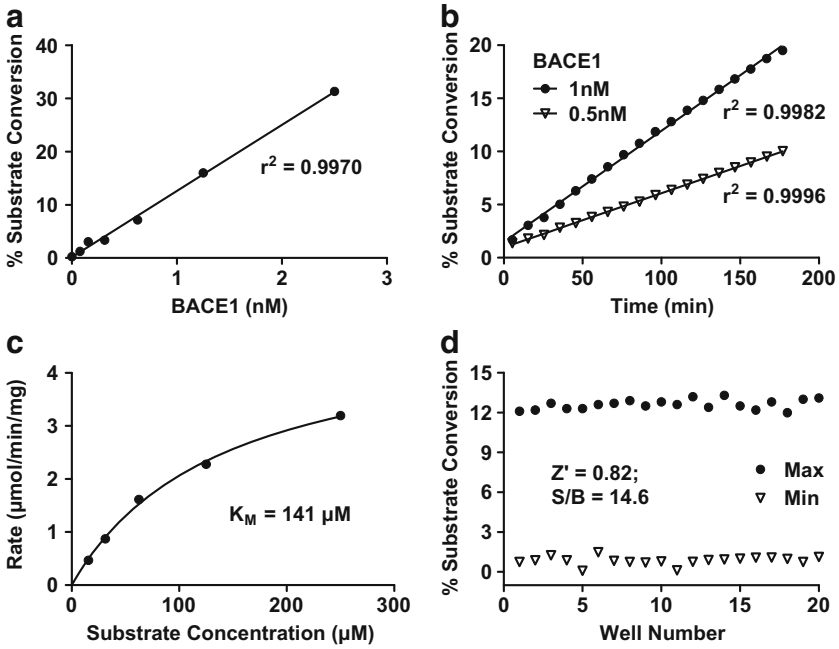

Fig. 2 Biochemical characterization of BACE1 using the MMSA. **a** Titration of BACE1 in the assay. **b** Time course of BACE1 enzyme reaction. **c** Michaelis–Menten kinetics plot for BACE1 with FAM-EVNLDAEF as the substrate. The above data were performed in duplicate. **d** Performance of mobility shift assay. S/B represents signal-to-background ratio

(ratio of signal to background) of MMSA (S/B = 14.6, Fig. 2d) was higher than that in HTRF (S/B was about 2) assay [14]. We also estimated the tolerance of the MMSA to DMSO by comparing the substrate conversion at various DMSO concentrations, and no significant effect was observed with final DMSO concentration up to 4% (data not shown). In terms of cost, MMSA and CE-MS need additional separation chips or columns, whereas FRET or HTRF need to obtain complicated substrates or beads. Taken together, the following pilot screening would be conducted at 1 nM of BACE1 and 5 μM of substrate for 1-h incubation.

Validation of the mobility shift assay with known inhibitors

To validate the MMSA, two well-known pioneers in commercial research, AZD3839 and AZD3293, were selected for the inhibition and kinetic studies. AZD3839 is a potent and selective BACE1 inhibitor (K_i = 26.1 nM) that could effectively reduce brain and CSF Aβ levels in preclinical and clinical studies [25, 26]. AZD3293 is a potent (IC_{50} = 0.6 ± 0.04 nM), highly permeable, orally active, and blood–brain-barrier-penetrating BACE1 inhibitor with unique slow off-rate kinetics [27, 28]. In

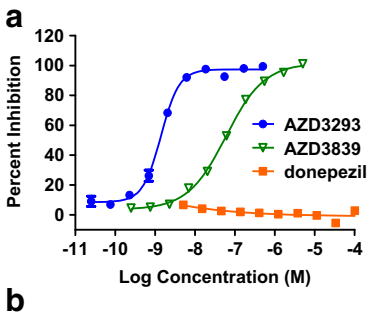

Fig. 3 Inhibition studies of known inhibitors against BACE1. **a** Dose–response curves for compounds inhibition of BACE1 in the MMSA. The mean IC_{50} values and standard deviations derived from four parallel determinations are listed. **b** Potencies of compounds to inhibit BACE1 in MMSA, FRET, and HTRF assays

b

nM	MSA	FRET	HTRF	CE-MS
AZD3293	1.4 ± 0.2 (IC_{50})	0.6 ± 0.04 (IC_{50}) [27]	ND	ND
AZD3839	59.8 ± 2.7 (IC_{50})	ND	26.1 (K_i) [25]	ND
donepezil	>100,000 (IC_{50})	171 ± 3 (IC_{50}) [12]	ND	>100,000 (IC_{50}) [18]

ND, no data available.

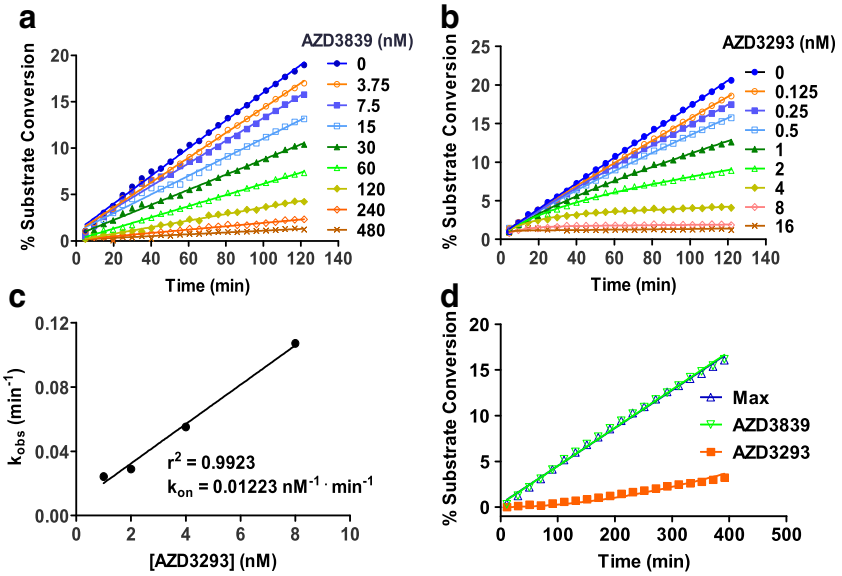

Fig. 4 Kinetic studies of known inhibitors against BACE1. **a** Time courses of BACE1 activity in the presence of different concentrations of AZD3839. **b** Time courses of BACE1 activity in the presence of different concentrations of AZD3293. **c** Plot of k_{obs} as a function of inhibitor concentration for the slow binding inhibitor AZD3293. **d** Reversibility assays with BACE1 and inhibitors using spin column method. The data of reversibility assays were performed in duplicate

the inhibition studies, the resulting IC_{50} values of two inhibitors (AZD3839 and AZD3293) determined by the MMSA were 59.8 ± 2.7 nM and 1.4 ± 0.2 nM, respectively (Fig. 3a), which were in very good agreement with those reported by FRET and HTRF methods (Fig. 3b).

Furthermore, we adopted kinetic studies to validate the assay in a continuous manner, which was important for assessing inhibitor efficacy and safety. A series of inhibition progression curves were generated in the presence of BACE1 inhibitors at different concentrations to determine the k_{on} values (Fig. 4a–c). The k_{off} values were determined using progression curves of the diluted BACE1 samples (Fig. 4d). As shown in Fig. 4a, AZD3839 displayed a simple linear product-versus-time relationship, indicating that it was a fast on-rate inhibitor for BACE1. For the reversibility assay, Fig. 4d depicted the recovery of enzyme activity following dilution of the purified enzyme–inhibitor complex with substrate. Dilution of BACE1–AZD3839 complex resulted in a rapid recovery of full enzyme activity, demonstrating that it was a fully reversible inhibitor. In contrast, AZD3293 displayed a quasi-linear relationship with time in the early stage, which was converted to a different linear relationship in the late stage, revealing that it was a slow on-rate inhibitor (Fig. 4b). Plot of k_{obs} as a function of AZD3293 concentration yielded a linear trend with the slope equal to k_{on} value (0.01223 nM^{-1}·min^{-1}), suggesting a competitive tight-binding mechanism (Fig. 4c). Moreover, AZD3293 showed a markedly slow off-rate mechanism with k_{off} value of

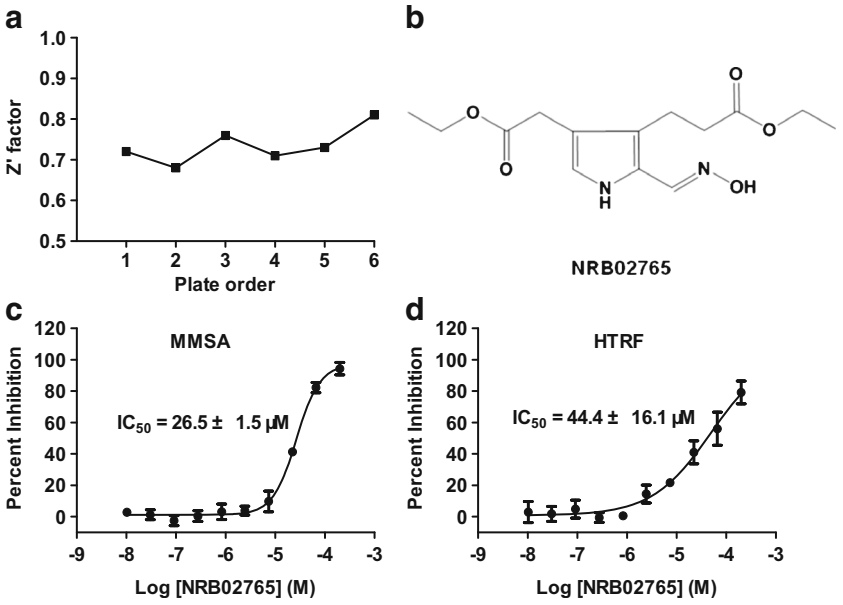

Fig. 5 Pilot screening of novel BACE1 inhibitors. **a** Variation of *Z′* factor value across plates. **b** Structure of the hit NRB02765. **c** Dose–response curves for compound inhibition of BACE1 in the MMSA. **d** Dose–response curves for compound inhibition of BACE1 in the HTRF assay. The mean IC_{50} values and standard deviations derived from four parallel determinations are listed

0.0136 min^{-1} (Fig. 4d). The calculated $t_{1/2}$ was 8.5 h, which was in good agreement with the estimated approximately 9 h from the discontinuous assay in the literature [27]. In that report, the authors also mentioned that the in vitro unique slow off-rate of AZD3293 might have translated into a prolongation of the on-target effect in vivo. Therefore, our assay was valuable for the kinetic studies of drug candidates with special mechanisms, which might provide some indications to the in vivo efficacies of these drugs. Thus, the inhibition and kinetic parameters of known inhibitors determined by our novel assay were comparable with those reported by common fluorescence-based assays.

Besides the two known BACE1 inhibitors, donepezil, an interesting acetylcholinesterase inhibitor, was also tested in our assay. Previous studies showed that it had an IC_{50} of 171 ± 3 nM in the FRET assay [12], but no inhibition was observed in the CE-MS assay using unlabeled substrate [18]. They gave a possible explanation for the observed inhibition in the FRET assay to be due to the concentration-dependent quenching of the primary fluorophore after hydrolysis by donepezil or other inhibitor–substrate interactions. In our research, even if the concentration of donepezil was up to 100 μM, no inhibitory effect was detected (Fig. 3a), which was consistent with that of the CE-MS assay. Such kind of potential interference of fluorescent compounds will be minimized in our MMSA system.

Application of the mobility shift assay for the pilot screening of novel inhibitors

In order to evaluate the performance of our MMSA, we screened a 900-compound Maybridge HitFinder™ library in duplicate in a 384-well format. The results of pilot screening showed that the MMSA was robust, with Z' factor values ranging between 0.68 and 0.81, and a mean value of 0.74 for the six plates (Fig. 5a). A novel hit (code NRB02765), ethyl 3-[4-(2-ethoxy-2-oxoethyl)-2-((hydroxyiminomethyl)-1H-pyrrol-3-yl]propanoate, was identified (Fig. 5b). Dose–response analysis displayed that it had an IC_{50} value of 26.5 ± 1.5 μM (Fig. 5c). The inhibitory effect of the hit was further confirmed in the HTRF assay, with IC_{50} value of 44.4 ± 16.1 μM (Fig. 5d). It is worth noting that all the compounds had no significant autofluorescence interference in the MMSA system during the pilot screening.

Conclusions

In this study, a new platform was developed, validated, and applied for the screening of BACE1 inhibitors in both kinetic and endpoint modes. By using the in vitro assay, one novel hit (IC_{50} = 26.5 ± 1.5 μM) was identified from a 900-compound library. Thus, the MMSA can be used not only for discovering additional BACE1 inhibitors with lower autofluorescence interference in endpoint manner but also for further studies on the mechanism of inhibitors under kinetic mode.

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Compliance with ethical standards This paper does not contain any studies with human participants or animals performed by any of the authors.

Conflict of interest The authors declare that they have no conflict of interest.

References

1. Prince M, Comas-Herrera A. Knapp M, Guerchet M, Karagiannidou, M. World Alzheimer Report 2016. Alzheimer's disease international. 2016. https://www.alz.co.uk/research/WorldAlzheimerReport2016.pdf/.
2. Alzheimer's association. Alzheimer's disease facts and figures. Alzheimers Dement. 2016;12:459–509.
3. Vassar R, Kuhn PH, Haass C, Kennedy ME, Rajendran L, Wong PC, et al. Function, therapeutic potential and cell biology of BACE proteases: current status and future prospects. J Neurochem. 2014;130:4–28.
4. Hartmann T, Bieger SC, Bruhl B, Tienari PJ, Ida N, Allsop D, et al. Distinct sites of intracellular production for Alzheimer's disease Aβ40/42 amyloid peptides. Nat Med. 1997;3:1016–20.
5. Hardy J, Selkoe DJ. The amyloid hypothesis of Alzheimer's disease: progress and problems on the road to therapeutics. Science. 2002;297:353–6.
6. Olsson F, Schmidt S, Althoff V, Munter LM, Jin S, Rosqvist S, et al. Characterization of intermediate steps in amyloid beta (Aβ) production under near-native conditions. J Biol Chem. 2014;289:1540–50.
7. Menting KW, Claassen JAHR. β-Secretase inhibitor; a promising novel therapeutic drug in Alzheimer's disease. Front Aging Neurosc. 2014;6:165. https://doi.org/10.3389/fnagi.2014.00165.
8. Ghosh AK, Osswald HL. BACE1 (β-secretase) inhibitors for the treatment of Alzheimer's disease. Chem Soc Rev. 2014;43:6765–813.
9. Cummings J, Morstorf T, Lee G. Alzheimer's drug-development pipeline: 2016. Alzheimers Dement. 2016;12:222–32.
10. Mancini F, Simone AD, Andrisano V. Beta-secretase as a target for Alzheimer's disease drug discovery: an overview of *in vitro* methods for characterization of inhibitors. Anal Bioanal Chem. 2011;400:1979–96.
11. Gruninger-Leitch F, Schlatter D, Kung E, Nelbock P, Dobeli H. Substrate and inhibitor profile of BACE (β-secretase) and comparison with other mammalian aspartic proteases. J Biol Chem. 2002;277:4687–93.
12. Mancini F, Naldi M, Cavrini V, Andrisano V. Multiwell fluorometric andcolorimetric microassays for the evaluation of beta-secretase (BACE-1) inhibitors. Anal Bioanal Chem. 2007;388:1175–83.
13. Manzenrieder F, Frank AO, Huber T, Dorner-Ciossek C, Kessler H. Synthesis and biological evaluation of phosphino dipeptide isostere inhibitor of human β-secretase (BACE1). Bioorg Med Chem. 2007;15:4136–43.
14. Kennedy ME, Wang W, Song L, Lee J, Zhang L, Wong G, et al. Measuring human β-secretase (BACE1) activity using

homogeneous time-resolved fluorescence. Anal Biochem. 2003;319:49–55.
15. Kornacker MG, Lai Z, Witmer M, Ma J, Hendrick J, Lee VG, et al. An inhibitor binding pocket distinct from the catalytic active site on human β-APP cleaving enzyme. Biochemistry. 2005;44:11567–73.
16. Mancini F, Andrisano V. Development of a liquid chromatographic system with fluorescent detection for β-secretase immobilized enzyme reactor on-line enzymatic studies. J Pharm Biomed Anal. 2010;52:355–61.
17. Simone AD, Mancini F, Cosconati S, Marinelli L, Pietra VL, Novellino E, et al. Human recombinant beta-secretase immobilized enzyme reactor for fast hits' selection and characterization from a virtual screening library. J Pharm Biomed Anal. 2013;73:131–4.
18. Schejbal J, Slezackova L, Reminek R, Glatz ZA. Capillary electrophoresis-mass spectrometry based method for the screening of β-secretase inhibitors as potential Alzheimer's disease therapeutics. J Chromatogr A. 2017;1487:235–41.
19. Yi X, Hao Y, Xia N, Wang J, Quintero M, Li D, et al. Sensitive and continuous screening of inhibitors of β-site amyloid precursor protein cleaving enzyme 1 (BACE1) at single SPR chips. Anal Chem. 2013;85:3660–6.
20. Ren Z, Tam D, YZ X, Wone D, Yuan S, Sham HL, et al. Development of a novel β-secretase binding assay using the AlphaScreen platform. J Biomol Screen. 2013;18:695–704.
21. Lo MC, Aulabaugh A, Jin G, Cowling R, Bard J, Malamas M, et al. Evaluation of fluorescence-based thermal shift assays for hit identification in drug discovery. Anal Biochem. 2004;332:153–9.
22. Harrison DJ, Fluri K, Seiler K, Fan Z, Effenhauser CS, Manz A. Micromachining a miniaturized capillary electrophoresis-based chemical analysis system on a chip. Science. 1993;261:895–7.
23. Perrin D, Fremaux C, Shutes A. Capillary microfluidic electrophoretic mobility shift assays: application to enzymatic assays in drug discovery. Expert Opin Drug Discov. 2010;5:51–63.
24. Copeland RA. Enzymes: a practical introduction to structure, mechanism, and data analysis. 2nd ed. New York: Wiley-VCH; 2000.
25. Jeppsson F, Eketjall S, Janson J, Karlstrom S, Gustavsson S, Olsson LL, et al. Discovery of AZD3839, a potent and selective BACE1 inhibitor clinical candidate for the treatment of Alzheimer disease. J Biol Chem. 2012;287:41245–57.
26. Quartino A, Huledal G, Sparve E, Lüttgen M, Bueters T, Karlsson P, et al. Population pharmacokinetic and pharmacodynamic analysis of plasma Aβ40 and Aβ42 following single oral doses of the BACE1 inhibitor AZD3839 to healthy volunteers. Clin Pharmacol Drug Dev. 2014;3:396–405.
27. Eketjall S, Janson J, Kaspersson K, Bogstedt A, Jeppsson F, Falting J, et al. AZD3293: a novel, orally active bace1 inhibitor with high potency and permeability and markedly slow off-rate kinetics. J Alzheimers Dis. 2016;50:1109–23.
28. Cebers G, Alexander RC, Haeberlein SB, Han D, Goldwater R, Ereshefsky L, et al. AZD3293: pharmacokinetic and pharmacodynamic effects in healthy subjects and patients with Alzheimer's disease. J Alzheimers Dis. 2017;55:1039–53.