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Inkjet printing-based β-secretase fluorescence resonance energy transfer (FRET) assay for screening of potential β-secretase inhibitors of Alzheimer's disease

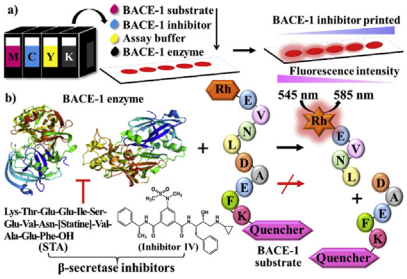
Jungmi Lee [1], Annie Agnes Suganya Samson [1], Joon Myong Song*

College of Pharmacy, Seoul National University, Seoul, 08826, South Korea

HIGHLIGHTS

- An inkjet printing-based fluorescence assay was developed for high throughput screening of β-secretase inhibitors using FRET peptide substrate.
- The BACE-1 peptide substrate printed on parchment paper was effectively cleaved by BACE-1 which printed on the same spot.
- The inkjet-printing-based BACE-1 inhibitory assay revealed inhibitory effects of inhibitor IV and STA on BACE-1.
- The inkjet printing-BACE-1 inhibitory assay revealed that the consumed amount of the sample was reduced by 1.4×10^3 times.
- The inkjet printing-based inhibitory assay enabled in a high throughput format, and it is a reliable for BACE-1 inhibitor screening.

GRAPHICAL ABSTRACT



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ABSTRACT

Amyloid-β (Aβ) is generated by proteolytic processing of amyloid precursor protein (APP) by beta-secretase (BACE-1) and gamma-secretase. Amyloid-β is responsible for the formation of senile plaques in Alzheimer's disease (AD). Consequently, inhibition of β-secretase (BACE-1), a rate-limiting enzyme in the production of Aβ, constitutes an attractive therapeutic approach to the treatment of AD. This paper reports an inkjet printing-based fluorescence assay for high throughput screening of β-secretase inhibitors achieved by employing a BACE-1 FRET substrate (Rh-Glu-Val-Asn-Leu-Asp-Ala-Glu-Phe-Lys-Quencher). This peptide substrate is known to be a readily available and suitable substrate for proteolytic activity, and it has high affinity to BACE-1. The BACE-1 peptide substrate printed on parchment paper was effectively cleaved by BACE-1, which was printed on the same spot. The amount of enzyme and substrate required for this inkjet printing-based BACE-1 assay can be less than $1.4x10^3$, permitting the evaluation of inhibitor activity with femtomolar potency. The inkjet-printing-based BACE-1 inhibitory assay revealed inhibitory effects of inhibitor IV and STA on BACE-1 with an IM_{50} of 1.00×10^{-15} mol and 1.01×10^{-14} mol, respectively. These data confirm that both BACE-1 inhibitors (inhibitor IV and STA)

* Corresponding author.
E-mail address: jmsong@snu.ac.kr (J.M. Song).
[1] These authors contributed equally.

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actively inhibited the BACE-1 proteolysis of BACE-1 substrate on parchment paper. It important to note that the number of mole of BACE-1-substrate and enzyme utilized in the printing-based enzymatic assay are $1.4x10^3$ smaller than the amount used in the conventional well-plate assay. The inkjet printing-based inhibitory assay constitutes a versatile high throughput technique and the IM_{50} values of the inhibitors were obtained with satisfactory reproducibility, suggesting that this inkjet-printing BACE-1 inhibitory assay could be quite suitable for the screening of new potential BACE-1 inhibitors for AD.

1. Introduction

Alzheimer's disease (AD) is characterized by the prevalence of senile plaques, as well as neuronal and synaptic loss, leading to severe memory loss and neuron cell death. Senile plaques consist predominantly of fibrils of amyloid-β (Aβ), a 39 to 43 amino acid peptide generated by proteolysis of amyloid precursor protein (APP) [1]. An excess level of amyloid-β (Aβ) in the brain is closely related to the pathogenesis of AD [2–4]. To produce amyloid-β, amyloid precursor protein, a type I single-transmembrane glycoprotein, is sequentially cleaved by two aspartyl proteases: β- and γ-secretase [5]. Due to their essential role in the generation of Aβ, both β- and γ-secretase are considered to be prime therapeutic targets [6–9]. In some cases, early onset of familial AD can be characteristic of a "Swedish" mutation in the amyloid precursor protein (APP). Mutation in the APP intensely enhances the cleavage of this protein by β-secretase (BACE1) [10–14]. β-secretase is a critical enzyme that catalyzes the production of the β-amyloid peptide (Aβ). Genetic and pathological evidence has led to a therapeutic approach that focuses on the inhibition of β-secretase (BACE1-β-site APP Cleaving Enzyme 1, EC 3.4.23.46) for the treatment of AD [15,16]. The objective of this present work was to develop a reliable assay for the evaluation of BACE-1 inhibition activity through an inkjet printing method that utilizes efficient sample processing, an easily available and suitable substrate, and consumption of a lesser sample volume of nL range, which will facilitate screening of a number of candidate compounds. High-throughput screening (HTS) is one of the techniques employed in the drug design and screening process. HTS is very efficient in the assessment of biological activities of candidate compounds. Recently, studies have emerged to overcome the challenges and enables to study AD through HTS. For example, HTS assay-based on tissue microarray (TMA) in the assessment of fibrinogen extravasation [17], large scale microfluidic chip in 96-well format for HTS-based image analysis of different populations of *Caenorhabditis elegans* [18], siRNA screening of the kinome identifies kinases involved in Ad [19] and antibody-mimetic 2D peptoid nanosheets [20] were utilized to study AD.

The drug discovery process has both advantages and disadvantages. Consequently, the current failure rate of finding and screening BACE1 inhibitors has motivated researchers to search for alternative small molecules with therapeutic potential for reducing risk factors associated with AD. The modulation of beta-secretase (BACE-1) activity is being investigated as a potential therapeutic target for treating AD. Substrate-based inhibitors usually exhibit high potency and selectivity. However, peptide substrate-based screening using manual pipetting unavoidably entails inaccuracy of delivered reagent volume, as well as reagent volume of nL level. Thus, it requires considerable subsequent modifications. Overall, a combinatorial approach that sensitively incorporates different strategies may enable its successful application in the future screening of BACE1 inhibitory drugs. To address some of these problems, this paper reports an inkjet printer-based fluorescence assay for evaluating BACE-1 inhibitors. In order to validate the new method with BACE-1 substrate, FRET containing the Swedish mutant peptide sequence (Glu-Val-Asn-Leu-Asp-Ala-Glu-Phe-Lys) was used as a reference for determining β-secretase activities/inhibition using an inkjet printer. BACE-1 substrate (Rh-Glu-Val-Asn-Leu-Asp-Ala-Glu-Phe-Lys-Quencher) is cleaved by BACE-1 enzyme at Leu-Asp (restriction site), and it consists of a fluorescent donor (Rhodamine (Rh) derivative) and a quenching acceptor. The weakly fluorescent substrate (intact BACE-1 substrate) becomes highly fluorescent upon enzymatic cleavage. The increase in fluorescence is linearly related to the rate of proteolysis. BACE-1 FRET assays were carried out using 384-well-plates and the inkjet printing method. Fig. 1a summarizes the FRET peptide-based fluorescent assay for measuring β-secretase inhibition using an inkjet printer. Briefly, BACE-1 peptide substrate (M cartridge), BACE-1 enzyme (K cartridge), assay buffer (Y cartridge), and BACE-1 inhibitors (inhibitor IV and STA) (C cartridge) were sequentially printed on parchment paper according to Song et al. [20]. Hence, this printing method allows high throughput verification. Moreover, this fluorescence assay is used to determine β-secretase inhibition activity based on different doses of β-secretase inhibitors printed simultaneously.

2. Materials and methods

2.1. Materials

BACE-1 (β-Secretase) Human Protein (#P2947) and BACE-1 substrate (Rh-EVNLDAEFK-Quencher; #P2986) were purchased from Thermo Fisher (USA). β-secretase inhibitor IV (565788) was purchased from Merck (Germany). Peptide-based β-secretase inhibitors-STA (Lys-Thr-Glu-Glu-Ile-Ser-Glu-Val-Asn-[Statine]-Val-

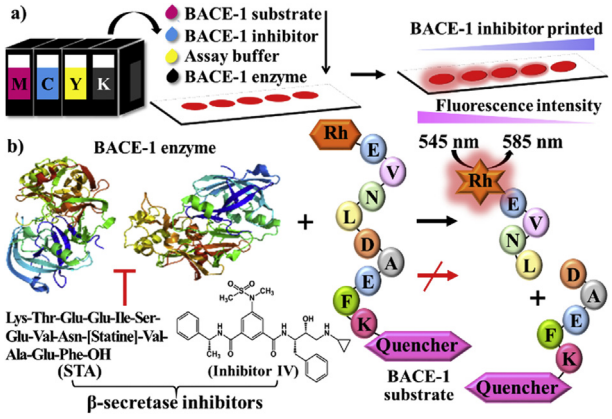

Fig. 1. a) A schematic representation of FRET peptide-based fluorescent assay to determine β-secretase inhibition using an inkjet printer. b) Illustration of the activity of β-secretase enzyme and β-secretase inhibitor with FRET peptide, which was printed on parchment paper using inkjet printing. Peptide cleavage and fluorescence recovery at each reaction spot were detected.