Short Communication:

Computer-Aided Discovery of Pentapeptide AEYTR as a Potent Acetylcholinesterase Inhibitor

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Abstract: *One of the key targets in the drug development for potential Alzheimer's disease (AD) therapeutics is the search for acetylcholinesterase enzyme (AChE) inhibitors. Very recently, a pentapeptide AEYTR was reported as a potential inhibitor for AChE. The peptide was identified in a retrospectively validated virtual screening campaign, which was subsequently followed by 10 ns molecular dynamics (MD) simulations. The study aimed to characterize the structure and identify* in vitro *of AEYTR peptide as a potent acetylcholinesterase inhibitor. This article presents the structure characterization and the* in vitro *examination of the peptide as an AChE inhibitor, followed by MD simulations for 100 ns. The results show that the pentapeptide is a potent AChE inhibitor with an* IC_{50} *value in the picomolar range and stabilizes the enzyme during MD simulations.*

Keywords: *acetylcholinesterase; short peptide; computer-aided discovery*

■ INTRODUCTION

Identification of a potential drug with high confidence is the main challenge of computer-aided drug discovery nowadays [1]. Some combined approaches, also by incorporating machine learning (ML) techniques, were introduced to optimize the confidence in hits identification [2–7]. The structure-based virtual screening (SBVS) approach was initially built with molecular docking simulations as the backbone tool [8-9]. However, the prediction ability of the developed SBVS protocols using solely molecular docking required major improvements [10-11]. In 2007, Marcou and Rognan [12] introduced molecular interaction fingerprints (IFP) to re-score the docking poses. Some SBVS campaigns successfully increased the prediction ability by combining the molecular docking and the IFP identifications [2-3,13-15]. On the other hand, a combination of pharmacophore-based approaches with the IFP identifications could also significantly increase the SBVS prediction ability [4]. Since the IFP identification developed by Marcou and Rognan [12] required proprietary libraries, we developed similar software in Python called PyPLIF in 2013 [16-17]. Instead of IFP, PyPLIF produces Protein-Ligand Interaction Fingerprints (PLIF) as the output [16]. In combination with molecular docking simulations, a newly introduced descriptor derived from PLIF called ensemble PLIF (ensPLIF) could increase the SBVS prediction ability to identify estrogen receptor alpha (ERα) ligands with *F*-measure and the accuracy values of 0.769 and 0.993, respectively [6]. This highly predictive SBVS protocol could be reached by employing a machine learning approach, namely Recursive Partitioning and Regression Trees (RPART) [18-19]. Notably, the combined method could also provide strong suggestions of molecular determinants in protein-ligand binding [6,19].

Following the success story of developing a highly predictive SBVS protocol to identify ERα ligands [6,19], we developed SBVS protocols to identify inhibitors for acetylcholinesterase (AChE) [20]. The enzyme was selected as the drug target of interest due to its practicality for *in vitro* verification and its important roles in Alzheimer's Diseases (AD) [21]. The protocol employing ensPLIF was reported as the best SBVS

protocol with the *F*-measure and the accuracy values of 0.413 and 0.988 [20]. On the other hand, we have also developed databases of short peptides readily for SBVS campaigns [22]. SBVS campaigns to discover potent AChE inhibitors on some short peptides [22] equipped with the knowledge obtained from the literature review [21] and the retrospectively validated SBVS protocol [20] identified four tetrapeptides (i.e., AEKY, AERW, AEYQ, and AEYT) and a pentapeptide AEYTR as potential AChE inhibitors [23]. Short molecular dynamics (MD) simulations of 10 ns were used to virtually confirm the discovery of AEYTR as a potential AChE [23]. Therefore, *in vitro* verification of the virtually discovered AEYRT as a potent AChE inhibitor has become of timely and considerable interest.

In this article, we present the results of longer 100 ns MD simulations to show that the enzyme stabilizations by the peptide could be reached and maintained during the simulations. Following the MD simulations, the *in vitro* test of the peptide as an AChE inhibitor and the structure elucidation of the peptide AEYTR are also presented. The structure characterization was used to confirm the identity of the peptide and the *in vitro* test of the peptide in determining the potential AChE inhibitor.

■ EXPERIMENTAL SECTION

Materials

The peptide AEYTR (MW = 665.35 g/mol) with a purity of 95% was purchased from 1st Base Laboratories (Singapore). The material used for structure characterization using NMR 500 MHz was D_2O. The materials used for AChE inhibitory assay were the assay kit (Cat. #KA1607) purchased from Abnova (Taiwan) containing reagents including acetylthiocholine, assay buffer, 5,5′-dithio-bis (2-nitrobenzoic acid) (DTNB) and acetylcholinesterase; donepezil p.a. (Santa Cruz) as the positive control; ddH_2O and phosphate buffer saline (PBS). The materials used for MD simulations were the YASARA scene input file for MD simulations obtained from Prasasty and Istyastono [23] and the YANACONDA macro file to perform MD simulations in YASARA-Structure [24].

Instrumentation

The instruments to perform structure characterization were ^1H and ^{13}C NMR 500 MHz. (series JNM-ECZ500R, Japan). The instrument to perform *in vitro* assay was a Tecan™ Infinite® 200 Pro microplate reader (Switzerland). The instrument to run MD simulations was a GOLD virtual private server (VPS) provided by Rumah Web Indonesia (https://www.rumahweb.com/vps-indonesia/) with Ubuntu Linux 14.04.5 as the operating system, 8 GB RAM, and 8 virtual cores of 6.4 GHz each. YASARA-Structure version 19.9.17 to perform MD simulations [24] was installed in the VPS. A working station with Intel® Pentium® Silver N5000 as the CPU and 4 GB memory and Windows 10 Home as the operating system were used as the computer client to communicate and to control the VPS. PyMOL version 2.3.4 was installed in the working station to assist the visual inspection of the MD simulation results [25]. Additional important software used in this research were PLANTS version 1.2 [26-27], SPORES 1.3 [28], and PyPLIF [16-17].

Procedure

Molecular dynamics simulations

The MD simulations were performed by employing the macro *md_run.mcr* in YASARA-Structure in the VPS [23-24]. The complex resulted from the virtual screening obtained from Prasasty, and Istyastono [23] was used as the input file. The module "Clean" in YASARA-Structure was then used to add hydrogens and other missing atoms. Simulation cell was defined as 10 Å around all atoms with periodic boundary conditions. AMBER14 was used as the force field for the protein, GAFF2, and AM1BCC for the peptide AEYTR, and TIP3P for water [24]. The cut-off for the Van der Waals force calculation was set at 8 Å, and the Particle Mesh Ewald (PME) was set without cut-off for electrostatics [24]. The "Cell neutralization" module in YASARA-Structure was then run to add explicit water molecules to the cell at pH 7.4 and subsequently perform energy minimizations using the steepest descent method followed by simulated annealing minimizations of the

solvent to obtain the default density of the system, i.e., 0.997 g/cm^3 [24]. In total, 19106 explicit water molecules were employed in the system. After the steepest descent and simulated annealing minimizations to remove clashes, the simulation was run for 100 nanoseconds. The equations of motions were integrated with multiple time steps of 1.25 fs for bonded interactions and 2.5 fs for non-bonded interactions at a temperature of 298 K and a pressure of 1 atm (NPT ensemble) using algorithms described in detail previously [24]. After inspection of the RMSD of the protein backbone atoms as a function of simulation time, the first 5 ns were considered as equilibration time.

The results were then analyzed using the default macro *md_analyze.mcr*, and the snapshots were converted to pdb files using the default macro *md_convert.mcr* [24]. The stability of the AChE backbone atoms was analyzed by examining the deviation of the root-mean-square deviation (RMSD) values in every 5 ns of the simulation time [23,29]. The stability of the AEYTR hydrophobic interaction to the residue F^{331} of the AChE [20] was analyzed by examining the percentage of the interaction bitstrings in every 5 ns of the simulation time. The interaction bitstrings were identified by employing SPORES1.3, followed by PLANTS1.2 and PyPLIF for all AChE-AEYRT complexes converted from the snapshots. The interaction was considered stable if the percentage is equal to or more than 87.8% [20].

In vitro *verification*

The assay to measure AChE activity followed to AChE kit protocol (Cat. #KA1607) [30]. The reaction mixture was prepared in the 96-well microplates, consisted of 5 mL of 3 mM 5,5'-dithio-bis (2-nitrobenzoic acid), 5 mL of 75 mM acetylthiocholine iodide, 110 mL 0.1 M sodium phosphate buffer (pH 7.5) as working reagents and 120 mL of peptide solution as stock samples at different concentrations (25, 50, 100, 250, 500 ppm or 36, 75, 150, 376, 751 μM) and positive control (donepezil). Ten microliters of the AChE enzyme at a concentration of 0.25 U/mL was then added to the reaction mixture. The 200 μL water and 200 μL calibrator were separately transferred into wells of clear bottom 96-well plates, and 10 μL peptide samples were added per well in separate wells. The 190 μL of working reagent was freshly prepared and transferred to all sample wells in the microplate and immediately read at 412 nm every 2 min for 10 min at 25 °C in a Tecan infinite 200Pro microplate reader (Switzerland). The enzyme activity was determined by the following formula: AChE activity = $[(OD_{10} - OD_2)/(OD_{Cal} - OdH_2O)] \times n \times 200$ (U/L), where OD_{10} and OD_2 are the OD_{412nm} values of the sample at 10 min and 2 min, respectively. OD_{Cal} and ODH_2O are the OD_{412nm} values of the calibrator and water at 10 min., while n is the dilution factor (n = 40 for sample). The number "200" is the equivalent activity of the calibrator under the assay conditions. The percentage of enzymatic inhibition was calculated for the inhibitor and IC_{50} (ref-1) value as follows: $I\% = [I]/[I]+IC_{50} \times 100\%$, where [I] is the inhibitor concentration of AEYTR peptide at temperature 10 min, and IC_{50} value is the 50% inhibition concentration of the peptide at temperature 10 min. The peptide sample was assessed in triplicate. The extent of inhibition was expressed as the mean ± standard error of the 50% inhibition concentration (IC_{50}) of the enzymatic activity. To inspect the inhibition mode of AEYTR peptide, AChE activity was monitored with the concentration gradient of acetylthiocholine substrate in the absence or presence of 36 μM AEYTR peptide.

Structure elucidation

The NMR experiment was conducted in one dimension of ^{1}H, with field strength = 11.7473579 T (500 MHz), acq. duration = 1.74587904[s], spectrophotometer frequency = 500.15991521 MHz, offset = 7.0 ppm, set points = 16384, prescans = 1, resolution = 0.57277737 Hz, sweep = 9.38438438 kHz, sweep clipped = 7.50750751 kHz, total scans = 80, relaxation delay = 5 s. recvr. gain = 46, temp. get = 19.9 °C, spectral width = 8 μs, acquisition time = 1.74587904 s, flip angle = 45°, atn = 8.9 dB, pulse = 4 us. The one dimension of ^{13}C was prepared within the field strength = 11.7473579 T (500 MHz), acquisition duration = 0.82837504 s, spectrophotometer frequency = 125.76529768 MHz, offset = 100 ppm, set points = 32768, prescans = 4, resolution = 1.20718268 Hz, sweep = 39.55696203 kHz, sweep clipped = 31.64556962 kHz.

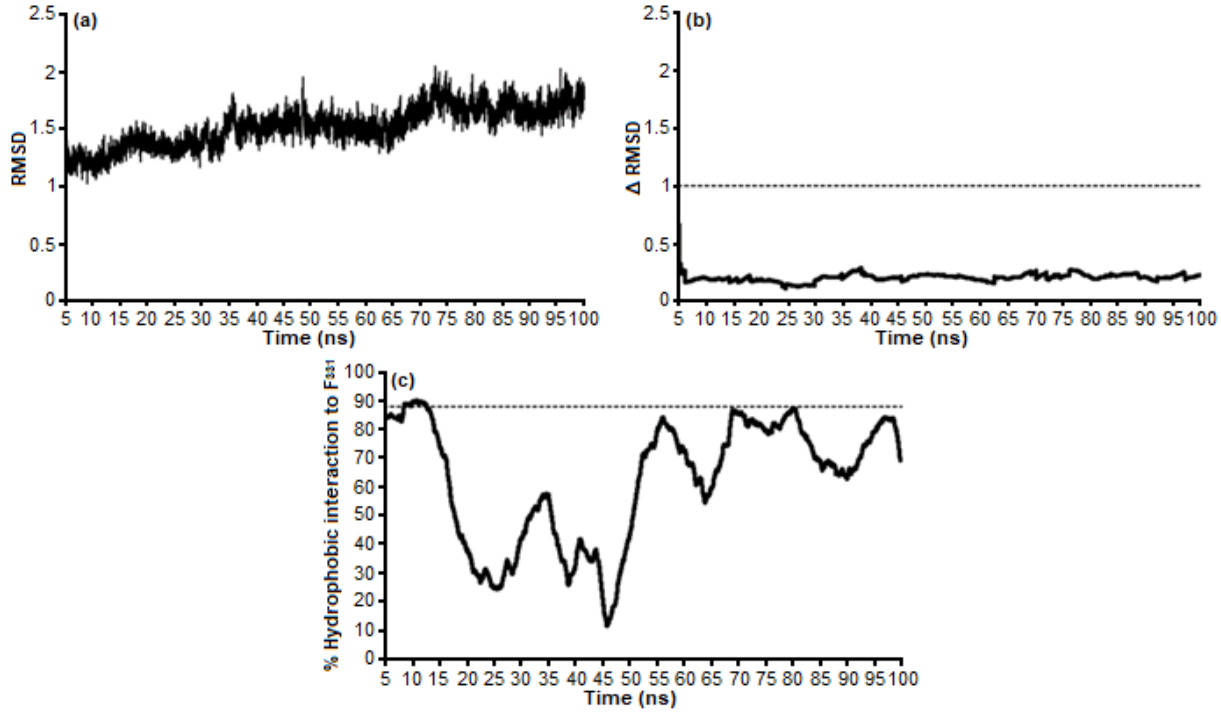

Fig 1. (a) The RMSD values of the AChE backbone atoms of the AChE during the production run of the MD simulations, (b) The average value of the RMSD deviation in every 5 ns (Δ RMSD) during the MD simulations, (c) The percentage of the hydrophobic interaction formation of AEYTR to F^{331} of AChE during the MD simulations

■ RESULTS AND DISCUSSION

Aimed to verify the activity of AEYTR as a potent AChE inhibitor, MD simulations for 100 ns and *in vitro* tests were performed. Structure elucidation of the purchased AEYTR was also performed to confirm the identity of the tested pentapeptide. The stability of the AChE backbone atoms and the hydrophobic interactions of AEYTR to F^{331} are presented in Fig. 1. The results of the *in vitro* experiments are presented in Fig. 2. The spectra resulted from the structure elucidation are presented in Fig. 3.

The backbone atoms of the AChE were reported stable since the beginning of the MD simulations (Fig. 1(a)). All delta RMSD values in each 5 ns are less than 1 Å (Fig. 1(b)) [23,29]. Liu et al. [29] suggested 10 ns MD simulations to explore the stability of protein-ligand binding modes. However, with the advances in computer power, it was recommended to perform 50 ns or even 100 ns MD simulations [31-32]. Therefore, the 10 ns MD simulations by Prasasty and Istyastono [23] were re-performed and extended to 100 ns. Besides the stability of the AChE backbone atoms, we were tempted to examine

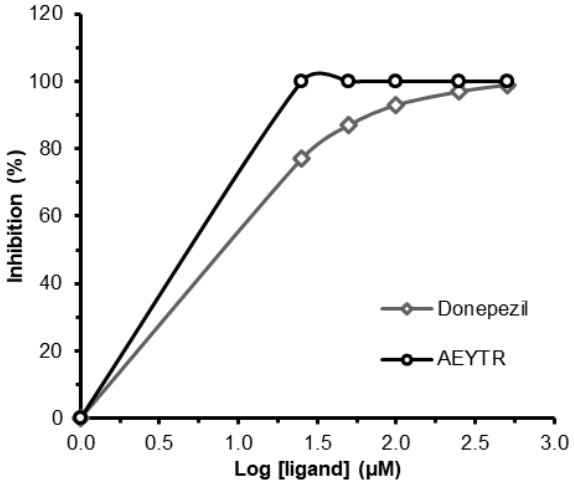

Fig 2. The drug response curves of the positive control donepezil and the peptide AEYTR as AChE inhibitors

the probability of the hydrophobic interaction formation between F^{331} of AChE and AEYTR during the simulation since the interaction was reported vital by Riswanto et al. [20]. In a decision tree incorporated in the retrospectively validated SBVS protocol to identify potent AChE inhibitors [20], the interaction must be equal to or more

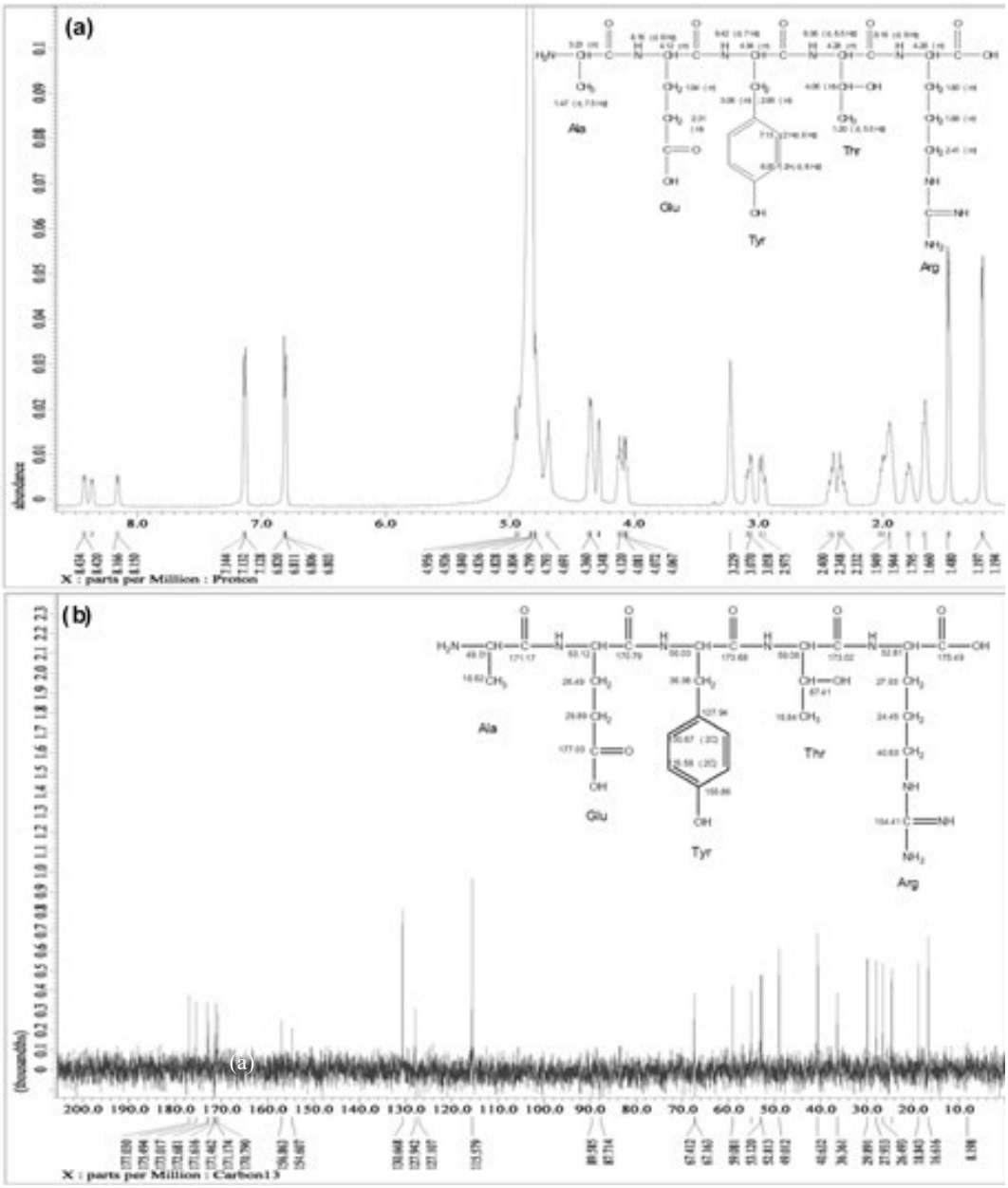

Fig 3. (a) The structure and ^{1}H-NMR chemical shift spectrum result of AEYTR, (b) The structure and ^{13}C-NMR chemical shift spectrum result of AEYTR

than 87.8%. During the MD simulations, by employing similar criteria by Liu et al. [29], the percentage was achieved from 8.46 ns to 12.64 ns (Fig. 1(c)). Notably, the results showed that although the peptide AEYTR has started to unbind from the F^{331} of the AChE after 12.64 ns (Fig. 1(c)), it could still maintain the stability of the enzyme (Fig. 1(b)). These results could be used for *in silico* explanation of the AEYTR inhibitory activity towards AChE.

The previously published peptides design by employing the retrospectively validated SBVS protocol constructed by Riswanto et al. [20] and the short peptide database by Prasasty and Istyastono [22] highly suggested pentapeptide AEYTR as a potential AChE inhibitor [21,23]. Recently, the *in vitro* verification of the previously computer-aided designed chalcones [20] was published [33]. These increased our confidence to perform *in vitro* verification on the pentapeptide

AEYTR. Notably, the *in vitro* experiments resulted in the IC_{50} values of donepezil as the positive control and AEYTR of 0.533 ± 0.051 and 0.462 ± 0.079 nM, respectively (Fig. 2).

The successful *in vitro* verification was required for the identity confirmation of the pentapeptide AEYTR. Therefore, structure elucidation was performed. In the ^{1}H-^{13}C-NMR spectrum, there were specific signals from alanine (Ala/A) appearing at δ_H/δ_C 1.47 (CH_3, d, 7.5 Hz)/16.67 (CH_3), 3.23 (CH, m)/49.01 and 171.17 (C=O) which forms amide with glutamic acid. Amino acid signals from glutamate (Glu/E) are seen at δ_H/δ_C 8.16 (NH, d, 8 Hz), 4.12 (CH, m), 1.94 (CH_2) and 2.31 (CH_2, m) and reinforced the presence of a carboxylic group at δ_C 177.03 and amide at δ_C 173.68. The presence of tyrosine (Tyr/Y) indicated the presence of NH group as amide (CONH) indicated by the presence of signals at 8.42 (NH, d, 7 Hz), 4.94 (CH, m), 3.06, 2.95 (m, CH_2), an aromatic group at δ_H/δ_C 7.13 (2x = CH, d, 8 Hz)/130.67 (2x = CH), 6.81 (2, = CH, d, 8 Hz) and 2C quaternary carbons at δ_C 127.94 and 155.86 and carbonyl of an amide which appeared at δ_C 173.02 (CONH). Residues threonine (Thr/T) and arginine (Arg/R) are shown in the presence of two amides (NH) that appeared at 8.36 (d, 6.5 Hz) and 8.16 (d, 8 Hz), and other signals that can be seen in Fig. 3. Thus, this compound has a structure as depicted above, and it is also strengthened by the data of the ^{1}H-^{13}C-NMR chemical shift values (Fig. 3).

■ CONCLUSION

The computer-aided designed AEYTR stabilized the enzyme AChE in the 100 ns MD simulations. The *in vitro* experiments verified the *in silico* results that AEYTR was a potent inhibitor for AChE with the IC_{50} value of 0.462 ± 0.079 nM. The structure elucidation confirmed the identity of the tested peptide.

■ AUTHOR CONTRIBUTIONS

EPI conducted the MD simulations, VDP conducted the *in vitro* experiments and the structure elucidation, EPI wrote the initial manuscript, EPI and VDP revised the manuscript. All authors agreed to the final version of this manuscript.

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