VITEXIN AS A POTENTIAL INHIBITOR FOR THE ACTIVATION OF MICROGLIAL CELLS: INSIGHTS FROM MOLECULAR DOCKING AND MOLECULAR DYNAMICS

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Abstract

Background: Overly-activated microglia are known to be the culprit in chronic neuroinflammation in the presence of excessive lipopolysaccharide (LPS). Hence, abundance of pro-inflammatory cytokines are produced which later cause toxic and death to neurons. Toll-like receptor 4 (TLR4)/MD-2 complex found on the cell surface of microglia is responsible for the attachment of LPS and activation of nuclear factor-κB (NF-κB) downstream signalling pathway. Albeit vitexin has been shown to possess anti-inflammatory property, however, little is known on its ability to bind at the TLR4/MD-2 complex of microglia as well as to be an antagonist for LPS.

Results: The molecular docking result shows that both vitexin and donepezil are able to bind at the close proximity of LPS binding site located at the TLR4/MD-2 complex with the binding energy of -4.35 and -9.14 kcal/mol, respectively. During molecular dynamic simulations, both vitexin and donepezil formed stable complex with TLR4/MD-2 throughout the 20 ns time length with the RMSD values of 1.9Å and 2.0Å, respectively. As for the RMSF, both compounds have the RMSF values of <1.2Å.

Conclusions: Taken together, our results suggest that vitexin can be a potential anti-neuroinflammatory drug candidate by acting as an antagonist for LPS at the TLR4/MD-2 complex.
Keywords: Vitexin, molecular docking, molecular dynamics, anti-neuroinflammatory, microglial cell.

Introduction
Neuroinflammation has been postulated by many to be the key player of neurodegenerative diseases (Spagnuolo, Moccia & Russo, 2018; Erkkinen, Kim & Geschwind, 2017). In chronic neuroinflammation, the overly-activated microglial cells have been identified to be the culprit in the progression of neurodegenerative diseases (Block et al, 2007; Subhramanyam et al, 2019). The overly-activated microglial cells have the tendency to excessively secrete a myriad of pro-inflammatory cytokines (e.g. interleukin (IL)-6, IL-1β and tumour necrosis factor-α (TNF-α)) upon triggered with its stimuli such as lipopolysaccharide (LPS).

The LPS will interact with the TLR4/MD-2 complex that can be found on the cell surface of microglial cells (Kim et al, 2007; Stewart et al, 2010; Ohto et al, 2012). The attachment of LPS onto the binding site of TLR4/MD-2 complex allows the induction of downstream signalling cascade (Švajger et al, 2013; Ain, Batool & Choi, 2020). This phenomenon will cause the activation of nuclear factor-κB (NF-κB) transcription factor that subsequently express the pro-inflammatory cytokines (Yang et al, 2020). The dysregulation of TLR4/MD-2 complex will cause the chronic neuroinflammation to establish and progress (Guo & Schluesener, 2007).

The inhibition of TLR4 signalling pathway is said to play a crucial role in the effective of therapeutic strategy in suppressing the undesirable amount of pro-inflammatory cytokines (Gao et al, 2017). Despite of a number of antagonist against TLR4/MD-2 complex has been developed and proceeded to clinical trials, however, none of these antagonists have shown a success in meeting the primary endpoint to reduce the patient’s mortality rate (Rice et al, 2010; Barochia et al, 2011).

Vitexin (apigenin-8-C-β-D-glucopyranoside) can be found in a number of medicinal plant species namely Ficus deltoidea (Abu Bakar et al, 2018), pearl millet (Gaitan et al, 1989) and bamboo (Wang et al, 2015) as one of the plants’ major active compounds. The compound also has been known to possess a number of pharmacological properties such as anti-inflammatory (Dong et al, 2013; Rosa et al, 2016) and neuroprotective effect (Nurdiana et al, 2018). In addition, vitexin has recently been explored on its potential to play a role in epigenetic activities (Yahaya et al, 2020). Albeit numerous studies have shown its ability to act as anti-inflammation and neuroprotection, however, the information on the ability of the compound to
bind at the LPS binding site on TLR4/MD-2 complex and hence acting as the antagonist for LPS is yet to be fully elucidated. Hence, the present study aimed to bridge the gap by performing molecular docking and molecular dynamics with vitexin against TLR4/MD-2 complex.

**Methodology**

**Receptor and Ligand Preparation**
The crystal structure of Toll-like receptor 4 (TLR4)/MD-2 protein complex [PDB ID: 3VQ2 with resolution of 2.48 Å (Ohto et al, 2012)] was retrieved from Protein Data Bank (https://www.rcsb.org/). The 3D structures of vitexin (PubChem CID: 5280441) and donepezil (PubChem CID: 5741) were retrieved from PubChem database in .sdf file format and were later converted into .pdb format via online (https://cactus.nci.nih.gov/translate/).

**Molecular Docking**
The polar hydrogen and Kollman partial atomic charge were assigned to TLR4/MD-2 protein complex by using AutoDock4 software (Morris et al, 2009) and saved as AutoDock readable file. Both ligands (vitexin and donepezil) were made flexible, torsion root was set free and the protein was kept rigid. The protein binding site was defined at Leu54, Lys89, Arg90, Lys91, Lys122, Ile124, Lys125, Lys128, Tyr131 and Lys132 as described by Kim et al (2007) and Ohto et al (2012) with the grid size of 50 Å x 50 Å x 50 Å and spacing of 0.558 Å. The dimension was set at x= -20312, y= -18.262, z= 23.949. Lamarckian genetic algorithm (Fuhrmann et al, 2010) was used in this process with the energy evaluation of 250000 and a total of 100 runs inside the binding site. The outcome from this docking was later analysed by using AutoDockTools software (Morris et al, 2009) and visualised by using Biovia Discovery Studio Visualizer.

**Ligand-Receptor Interaction Analysis**
The 2-dimensional (2D) and surface annotation of both ligand interactions with the protein were generated and analysed by using Biovia Discovery Studio Visualizer.

**Molecular Dynamics (MD) Simulations**
MD simulations for 20ns were carried out using Desmond Simulation Package (Schrödinger, LLC) (Bowers et al, 2006). The protein-ligand complexes were processed by the Protein Preparation Wizard Tool by using default parameters (Madhavi Sastry et al, 2013).
Transferable Intermolecular Interaction Potential 3 Points (TIP3P) has been selected as the solvent model with 10x10x10 Å orthorhombic box. The counter ions (Na\(^+\) or Cl\(^-\)) were added and OPLS\(_{2005}\) force field parameters were used (Shivakumar et al, 2010). The \textit{NPT} ensemble with a temperature of 300K as well as 1atm pressure were applied during the simulations. To mimic the physiological conditions, 0.15M of NaCl was added. The trajectories from the MD simulations were saved for every 50ps intervals for analyses of root-mean-square deviation (RMSD), root-mean-square fluctuation (RMSF) as well as the protein-ligand contacts.
Result

Molecular Docking Analysis

The binding energy of vitexin and donepezil towards TLR4/MD-2 complex was analysed by using AutoDockTools software (Morris et al, 2009). The grid box was set at the protein binding site as per described by Kim et al (2007) and Ohto et al (2012). The docking results for both ligands were clustered with the RMSD tolerance of 2.0 Å. The binding sites of vitexin on the TLR4/MD-2 complex is visualised in Figure 1.

![Molecular docking visualisation of (A) donepezil and (B) vitexin against TLR4/MD-2 complex by using Biovia Discovery Studio Visualizer. Both ligands docked at the binding pocket of the TLR4/MD-2 complex](image)

**Figure 1:** Molecular docking visualisation of (A) donepezil and (B) vitexin against TLR4/MD-2 complex by using Biovia Discovery Studio Visualizer. Both ligands docked at the binding pocket of the TLR4/MD-2 complex.
The AutoDockTools software generated the output file and log file for each complex. The binding mode with the least binding energy and RMSD were considered as the best binding mode. Donepezil binds to TLR4/MD-2 complex with the binding energy of -9.14 kcal/mol. On the other hand, vitexin binds to TLR4/MD-2 complex with the binding energy of -4.35 kcal/mol. The summary of the docking analysis for both donepezil and vitexin is listed in Table 1.

Table 1: Summary of docking analysis for Donepezil and Vitexin by using AutoDockTools software.

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</tr>
</thead>
<tbody>
<tr>
<td>Donepezil</td>
<td>37.35</td>
<td>-9.14</td>
<td>198.79</td>
<td>-10.93</td>
<td>-0.27</td>
<td>-0.89</td>
<td>1.79</td>
<td>-.89</td>
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<td>Vitexin</td>
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<td>-4.35</td>
<td>647.72</td>
<td>-7.33</td>
<td>-0.02</td>
<td>-3.71</td>
<td>2.98</td>
<td>-3.71</td>
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Ligand-Receptor Interaction Analysis

From Figure 1, both ligands were able to dock at the binding pocket of TLR4/MD-2 complex. At the binding pocket of TLR4/MD-2 complex, both ligands interact with various number of amino acids with their respective interaction bond. As shown in Figure 2, donepezil interacted with Cys25, Ile32, Ile52, Val61, Ile80, Phe121, Ile124, Tyr131, Arg132, Cys133, Phe151 and Ile153. On the other hand, vitexin is shown to interact with Cys25, Ile32, Ile46 and Ile52. The summary of their residues along with their respective bond distance (Å) and type of interacted bond can be found in Table 2.
Figure 2: 2D residues diagram analysis for (A) donepezil and (B) vitexin generated by Biovia Discovery Studio Visualizer.
Table 2: Summary of 2D residues diagram analysis for donepezil and vitexin generated by Biovia Discovery Studio Visualizer.

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Interaction Amino Acid Residue</th>
<th>Bond Distance (Å)</th>
<th>Type of Interacted Bond</th>
</tr>
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<tr>
<td><strong>Donepezil</strong></td>
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<td></td>
<td>Ile32</td>
<td>5.56 and 5.86</td>
<td>π-Alkyl and Alkyl</td>
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<td></td>
<td>Ile52</td>
<td>5.03</td>
<td>Alkyl</td>
</tr>
<tr>
<td></td>
<td>Val61</td>
<td>6.17</td>
<td>Alkyl</td>
</tr>
<tr>
<td></td>
<td>Ile80</td>
<td>4.25</td>
<td>π-Sulfur</td>
</tr>
<tr>
<td></td>
<td>Phe121</td>
<td>7.56</td>
<td>π-π stacked</td>
</tr>
<tr>
<td></td>
<td>Ile124</td>
<td>5.56</td>
<td>Alkyl</td>
</tr>
<tr>
<td></td>
<td>Tyr131</td>
<td>5.13 and 5.91</td>
<td>π-Alkyl and π-Alkyl</td>
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<tr>
<td></td>
<td>Arg132</td>
<td>6.74</td>
<td>Carbon Hydrogen</td>
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<tr>
<td></td>
<td>Cys133</td>
<td>4.29 and 4.58</td>
<td>Alkyl and π-Sulfur</td>
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<td></td>
<td>Phe151</td>
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<td></td>
<td>Ile153</td>
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<td>5.24 and 6.91</td>
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<td>Ile52</td>
<td>5.96 and 7.04</td>
<td>π-Sigma and π-Alkyl</td>
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Molecular Dynamics (MD) Simulations

MD simulations were carried out for 20ns by using Desmond Simulation Package. The value of RMS and RMSF as well as protein-ligand contacts were evaluated from the MD trajectories data.

**Figure 3:** RMSD (Å) of the Cα atoms of TLR4/MD-2 complex and donepezil against time (nsec). The left y-axis shows the variation in the TLR4/MD-2 complex RMSD against time. The right y-axis shows the variation in the donepezil RMSD against time.
Figure 4: Analysis of donepezil on atom-wise RMSF (upper panel) and residue-wise RMSF (lower panel) of donepezil with respect to TLR4/MD-2 complex.
Figure 5: RMSD (Å) of the Cα atoms of TLR4/MD-2 complex and vitexin against time (nsec). The left y-axis shows the variation in the TLR4/MD-2 complex RMSD against time. The right y-axis shows the variation in the vitexin RMSD against time.
The RSMD plot for donepezil-TLR4/MD-2 complex (Figure 3) depicted that the complex reaches its stability at 4ns. An average RMSD value of 2.0Å persisted until 17.5ns. However, the RMSD value begins to irregularly fluctuate until 19.5ns before it becomes stable again at 20ns. On the other hand, the RMSD plot for vitexin-TLR4/MD-2 complex (Figure 5) showed that the complex reaches its stability at 4.5ns. An average RMSD value of 1.9Å remained until the end of simulation period. These results indicate that both ligands are stably bound at the binding site of TLR4/MD-2 complex during the entire simulation period. However, vitexin showed the most stable binding towards the TLR4/MD-2 complex when compared with donepezil.

Figure 4 and 6 illustrate the residue-wise RMSF values of TLR4/MD-2 complex bound with donepezil and vitexin, respectively. In addition, the figures also illustrate the atom-wise RMSF of both ligands in correspond to the TLR4/MD-2 complex. During the 20ns simulation, most amino acid residues have an RMSF value of <3.5Å. Meanwhile, both donepezil and vitexin atoms showed a RMSF value of <1.2Å. The low RMSF values for binding site residues and both ligands’ atoms designate the binding stability between both ligands with TLR4/MD-2 complex.
**Discussion**

Microglia have become the subject of interest amongst researchers since the cells have been shown to be one of the major culprits in neurodegenerative diseases due to their ability to be the enhancer for neuroinflammation which eventually lead to the death of neurons (Hansen, Hanson and Sheng, 2017). In its normal state, microglia have the role in maintaining the homeostasis of neural environment, response towards any injury and tissue repair as well as influencing the brain development (Zhang et al, 2019). In responding towards the injury, microglia need to be stimulated by its stimuli such as LPS in order for the cells to be in their active state (Mantovani & Locati, 2013).

However, when microglia become overly-activated for a longer period of time, the cells have the tendency to excessively produce higher amount of pro-inflammatory cytokines (e.g. tumour necrosis factor-α (TNF-α), interleukin-6 (IL-6)) into its microenvironment (Alam et al, 2016). This excessive amount of pro-inflammatory cytokines will cause toxic towards the neurons and ultimately encourage the establishment and progression of neuroinflammation. Kim et al (2007) and Ohto et al (2012) have shown that the TLR4/MD-2 complex found on the surface of microglial cells is crucial for the recognition of LPS. The activation of microglia by LPS through TLR4/MD-2 complex has allowed the induction of the downstream canonical neuroinflammatory pathways such as nuclear factor-κB (NF-κB) signalling pathway (Mantovani & Locati, 2013; Shi et al, 2019). As the result, pro-inflammatory cytokines will be secreted and thus, contribute to the worsen of neurodegenerative diseases.

In light of the therapeutic strategy to block the activation of TLR4 which gives rise to the chronic inflammation (Hennessy, Parker and O’Neill, 2010), the present study has chosen vitexin due to its reported anti-inflammatory and neuroprotective properties (Dong et al, 2013; Rosa et al, 2016; Nurdiana et al, 2018). The U.S. Food & Drug Administration (FDA)-approved Alzheimer’s disease (AD) drug; donepezil, has been selected to compare the efficacy of vitexin in acting as inhibitor against TLR4/MD-2 complex of microglia. Donepezil has been shown to not only able to inhibit the cholinergic activity, the drug also revealed to have potent anti-inflammatory effects in AD patients as well as in LPS-treated animals (Tyagi et al, 2010; Yoshiyama et al, 2010). Furthermore, Hwang et al (2010) reported that donepezil managed to deactivate microglia independently of its acetylcholine (ACh) receptor.

In reference to Kim et al (2007) and Ohoto et al (2012) studies, they have reported that Leu54, Lys89, Arg90, Lys91, Lys122, Ile124, Lys125, Lys128, Tyr131 and Lys132 are the essential site for the LPS to bind in order for the microglia to be activated. Hence, in preparing for the molecular docking analysis, this binding site has been covered during the grid box...
setting. The results from this study show that both donepezil and vitexin are able to bind at the binding pocket of TLR4/MD-2 complex with the binding energy of -9.14 kcal/mol and -4.35 kcal/mol, respectively.

Albeit vitexin did not bind at the exact binding site of TLR4/MD-2 complex as mentioned by Kim et al (2007) and Ohto et al (2012), however, the compound bound at the proximity of the binding site. This will, later, cause a disturbance in the interaction of LPS with the amino acids located at binding site of TLR4/MD-2 complex only if the compound is given in pre-treatment manner. Conversely, donepezil managed to bind at Tyr131 residue of the binding site of TLR4/MD-2 complex. This translates that donepezil can potentially inhibit the binding of LPS and hence, prevent the activation of microglia and eventually reducing the amount of pro-inflammatory cytokines being produced.

Upon performing MD simulation, the study found that both donepezil- and vitexin-TLR4/MD-2 complexes managed to stably bind for a period of 20ns. Interestingly, vitexin-TLR4/MD-2 complex was able to stabilise much longer when compared with the donepezil-TLR4/MD-2 complex.

**Conclusion**

The results from the present study revealed that vitexin has the potential to be an inhibitor for LPS in the activation of microglia. The hindrance of LPS to bind at the binding site of TLR4/MD-2 complex will prevent the activation of microglia and thus, preventing the over production of pro-inflammatory cytokines which eventually allowing the neurons to thrive. Though donepezil showed better result in this study when compared with vitexin, however, donepezil has been shown to give adverse effects. In addition, the consumption of donepezil as part of Alzheimer’s disease treatment can only delay the progress of the disease, however, it does not cure the disease. Hence, a new inhibitor is much needed to overcome the situation. Future *in vitro* and *in vivo* studies are much needed to verify these results within this context.

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**Author’s contributions**
MAFY and MZM planned and conceptualised the experiments. MAFY carried out the experiments and verified by ARAB. MAFY, ARAB, JS, NN, MZ and MZM drafted and finalised the manuscripts. All authors read and approved the final version of the manuscript.

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**Availability of data and materials**
The datasets generated and/or analysed during the current study are not publicly available as the authors are currently using the datasets for the next study. However, the datasets are available from the corresponding author upon reasonable request.

**Ethics approval and consent to participate**
Not applicable.

**Consent for publication**
Not applicable.

**Competing interests**
The authors declare that no competing interests are exist.

**References**


the molecular mechanisms. Nat Rev Neurosci, 8, 57-69.


