**Original Article**

**Resveratrol inhibition of TNF-α and IL-1 for treatment of rheumatoid arthritis: from *In-Silico* to *In-vitro* elucidation**

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**Abstract:** Resveratrol, a traditional Chinese medicine, known for its anti-inflammatory effect via cytokine inhibition, has long been tested and used in treating rheumatoid arthritis (RA). Here in this study we are exploring *in silico* approaches to understand the mechanistic inhibition of TNFα and IL-1 cytokines using molecular docking and molecular simulation approach. The pathway analysis using GeneMania of these two cytokines showed us their importance in the network of protein involved in RA. The molecular docking showed mechanistic inhibition of TNF-α and IL-1 by resveratrol and showed us that TNF-α is a better suited target for resveratrol inhibition for RA treatment. The molecular dynamics simulations showed the complex of resveratrol and TNF-α to be stable over a run of five nano seconds in an environment close to *in-vivo* conditions. The study is corroborating resveratrol to be a potential RA drug. The IC50 of the resveratol was determined to be 0.65 μM.

**Keywords:** Resveratrol, rheumatoid arthritis, TNF-α, IL-1, docking, simulations, immunomodulator activity

**Introduction**

Rheumatoid arthritis (RA) an arthrophathy of unknown etiology is a common inflammatory disease [1-4]. The etiology of the disease is governed by multiple factors, among smoking, obesity, drinking and genetic factors are well documented [5, 6]. The activated innate immune cells in RA patients release a spectrum of pro-inflammatory mediators: tumor necrosis factor alpha (TNFα), interleukin 1 (IL-1) [7-9], these mediators have a progressive role in joint inflammation and tissue destruction by inducing the production of metalloproteinases [3]. The concentration of these two cytokines is high in synovial fluid and the plasma in RA patients [10, 11].

Targeting these two cytokines has been attempted, TNFα inhibitors, anti-TNF antibody, a soluble TNF receptor fusion protein and IL-1 receptor antagonist have tried and tested [12, 13]. Compounds of natural origin have been used to target cytokines for treatment of RA [14] the compound of our interest here is 3,5,4’-trihydroxystilbene also known as resveratrol, a plant phytoalexin [15, 16]. Resveratrol is known to be present in grape skin and red wine and has been reported to reduce superoxides, suppress carcinogenesis, angiogenesis, diabetes mellitus and inhibit inflammation by blocking TNFα and or IL-1 [17-20].

Computer aided drug designing (CADD) has become an important tool in drug discovery [21], and in this study we are using this approach to demonstrate the mechanistic inhibition of TNFα and IL-1 cytokines by resveratrol at atomic level. The molecular docking approach has been used for the atomic insight [22].

**Material and methods**

**Protein preparation and pathway analysis**

The TNFα and IL-1 cytokines proteins three dimensional (3D) structure were retrieved from PDB (http://www.pdb.org/). The TNF-α protein structure contains contained 147 amino acids ranging from 10-157, its PDB ID: is 2AZ5 [23]. IL-1 crystallographic structure has 154 amino acids ranging from 117-271, the PDB ID: 2ILA is assigned to it [24]. All the modifications prior to molecular docking were done using Discovery
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The SDF format was converted to PDF for further study using Discovery Studio.

Molecular docking analysis

Molecular docking analysis of the TNF-α and IL-1 cytokine proteins with resveratrol was done. The top interactions out of ten were studied for each protein. Autodock 4.2 [26] tool was used for the study, the tool uses binding free energy evaluation to find the best binding mode between the compound and the protein. All the visualization were performed in Pymol [27].

Molecular dynamics simulation

The top compound selected was subjected to Molecular dynamics Simulations (MDS) using Gromacs 4.5.3 package [28]. Root mean square deviation (RMSD) root mean square fluctuation (RMSF) was checked using gromacs inbuilt tools g_rms and g_rmsf were used for the respective analysis.

Bioactivity

Immunomodulator activity of resveratrol was evaluated using oxidative burst assay. The standard protocol of chemiluminescence on reaction mixtures contained neutrophils was followed. Heparinized blood of healthy volunteers (18-45 years age) was used to purify neutrophils by dextran sedimentation and density gradient centrifugation. Neutrophils were adjusted to their required concentration using Hank’s Balance Salt Solution containing Ca and Mg (HBSS++). 25 µl of neutrophil cells were incubated 25 µl of serially diluted resveratrol with concentrations ranging from 0.1 µM to 10 µM. Control wells had neutrophil and HBSS++ but no resveratrol. Cells were washed after 30 min incubation with resveratrol HBSS++. Cells were activated by

Table 1. The binding pattern of resveratrol with TNF-α and IL-1, number of interactions and distance calculated by pymol

<table>
<thead>
<tr>
<th>Target Protein</th>
<th>ΔG (Kcal/mole)</th>
<th>No. of Interactions</th>
<th>Distance (Å)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF-α</td>
<td>-4.95</td>
<td>Four</td>
<td>Resveratrol-ASP45 (2.1Å)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Resveratrol-GLN47 (3.0Å)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Resveratrol-ILE136 (2.3Å)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Resveratrol-ILE136 (3.0Å)</td>
</tr>
<tr>
<td>IL-1</td>
<td>-4.43</td>
<td>Four</td>
<td>Resveratrol-LEU40 (3.1Å)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Resveratrol-LEU74 (2.8Å)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Resveratrol-VAL140 (2.3Å)</td>
</tr>
</tbody>
</table>

Studio 3.5 Visualizer [25]. The pathway of these two proteins was tracked using GENEMANIA server (www.genemania.org).

Compound selection

Resveratrol or 3,5,4′-trihydroxystilbene, a natural compound was retrieved from PubChem compound database. The SDF format of the compound was received bearing CID 445154.
adding 25 µl Opsonized zymosan-A, followed by 25 µl along with HBSS++. The oxidative burst was monitored as chemiluminescence RLU with peak and total integral values set with repeated scans at 30 s intervals and 1 s points measuring time.

Statistical analysis

Results are expressed as mean % inhibition of five determinations at level of significance P 0.5** and P 0.05*** and is calculated using ANOVA.
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The pathway interaction profile of TNF-α and IL-1 derived using GeneMania database, web-based integrative software, gave us the network architecture of the connections of the two cytokines and other proteins of main importance, as shown in Figure 1. The modified crystallographic structures of TNF-α and IL-1 were subjected to molecular docking using AutoDock 4.2. The results generated were analyzed using Pymol, the measurement tool of the Pymol was also used to calculate the distances of the hydrogen bond, which are shown in Table 1. The first cytokine to be evaluated was TNF-α. The resveratrol and TNF-α complex, Figure 2A was selected from the ten complexes generated by the AutoDock suit based on the binding energy $\Delta G$ of -4.95 Kcal/mol. The complex is forming four hydrogen bonds with GLN47, ASP45 and two with ILE136 shown Figure 3A. The H$_{29}$ position of resveratrol is interacting with O of ASP45 and the bond formed between them has a distance of 2.1 Å. The N of GLN47 is forming a hydrogen bond of distance 3 Å with H at 27th position of resveratrol. The other two hydrogen bonds are formed by ILE136 with oxygen and H$_{27}$ of resveratrol, with a distance of 3 and 2.3 Å respectively. The second complex of resveratrol and IL-1, Figure 2B is the best of the top ten results generated with the $\Delta G$ of -4.43 Kcal/mol. This complex is also forming four hydrogen bonds with LEU40, ILE74, LEU79 and VAL140 as shown in Figure 3B, where LEU40, ILE74, LEU79 and VAL140 amino acids of IL-1 are forming hydrogen bond with O$_2$, H$_{27}$, H$_{29}$ and O$_3$ atoms of resveratrol respectively. The Hydrogen bond distance is 3.1 Å’ between LEU40 and O$_3$, 2.8 Å’ between ILE74 and H$_{27}$, 2.1 Å’ between LEU79 and H$_{29}$, 2.3 Å’ between VAL140 and O$_2$. This complex is the second...
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best based on the binding energy. The top complex of TNF-resveratrol was subjected to MDS for further analysis. Five ns run was performed on this complex (Figure 4), prior to the run the complex was energy minimized for a period of 1 ns and a position restraints simulation was performed using steepest decent integrator on a step-wise manner. The RMSD and RMSF of the complex was checked (Figure 5). The RMSD of the complex after 5 ns of MD simulation was showing a stable run at 1.25 Å, showing very slight variation and concluding the complex to be stable throughout the run. The RMSF of the protein is represented by black in Figure 6 showing the fluctuation throughout the atoms of the protein. g-covar and g-anaeig of gromacs utilities was used to obtain the trajectories. In Figure 7 the projection PC 1 vs. PC 2 of the top complex is shown, their free energy surface was plotted the depiction shows that the stability of the complex over the run is uniform over time. To analyses the immunomodulator activity of resveratrol, the human neutrophils isolated from healthy individuals were incubated with several concentration of resveratrol for Five minutes. The chemiluminescence was measured using laminol as probe (Table 2). Resveratrol possess average inhibitory effects in the range of 71.5, 64.1, 52.8, 22.5 and 4% on monocytes, respectively, at 10, 1, 0.5, 0.15 and 0.1 µM concentration. Figure 8 shows % inhibition results to be dose dependent. The minimum concentration of resveratrol to show immunemodulation is 0.15 μM and IC50 of 0.65 μM was determined.

Discussion
Natural products are a said to be a gold mine for treatment for arthritis [14, 15] and our work explores resveratrol (3,4’;5 trihydroxystilbene) a natural product of spermatophytes. The prod-

**Figure 7.** PC 1 vs. PC 2 of resveratrol-TNF complex.

**Table 2.** Immunomodulating inhibitory properties of resveratrol of neutrophil cells

<table>
<thead>
<tr>
<th>Compound</th>
<th>Con. (µM)</th>
<th>RLU mean ± S.D.</th>
<th>Inh. %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resveratrol</td>
<td>10</td>
<td>204.2 ± 0.6</td>
<td>71.5</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>313.5 ± 2.1</td>
<td>64.1</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>376.9 ± 12.9</td>
<td>52.8</td>
</tr>
<tr>
<td></td>
<td>0.15</td>
<td>380.1 ± 12.2</td>
<td>22.5</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>837.1 ± 23.6</td>
<td>4.0</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td>872.3 ± 83.6</td>
<td></td>
</tr>
</tbody>
</table>

Con. = concentration. Inh.% = inhibition%.

**Figure 8.** Eight concentration of the resveratrol (0.1 to 10 µM) showing the inhibitory effect on oxidative burst as measured by chemiluminescence. The experiments were repeated five times.
In-Silico insight into resveratrol targeting TNF-α and IL-1

Product has long been in use for treatment of RA and work explores its possible protein targets using advanced Insilico techniques. The important proteins that are involved in the network and have a role in RA are IL-6, NFKB1A, MAP3K7, TGFB3, and TRAF6 [29-33], confirm-

Figure 9. A. The hydrophobic interaction between the resveratrol and TNF-α protein target. B. The three-dimensional representation of the same plot generated using Pymol.
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The resveratrol-TNF-α interactions and the number of hydrogen bonds formed are displayed in Figure 9A. Resveratol-TNF-α has a ΔG of -4.95 Kcal/mol. The hydrophobic binding pocket in this case is of following amino acids TRP139, VAL90, GLN38, LEU92, PHE54, LEU72, ILE32, ILE74, ARG73, TYR80, VAL81, LEU40, TYR39 and PRO89. Figure 9B shows three dimensional mapping of the hydrophobic interaction between resveratol and TNF-α. The inhibition of this cytokine alone or in tandem with IL-1 is pivotal drug inhibiting strategy for RA. The pathway analysis is conclusively showing the role TNF-α and IL-1 in RA. The invitro mechanistic approach to validate the binding was beyond the scope of the article; however the immunomodilation was elucidated using oxidative burst assay. The preliminary invitro results of neutrophil cells with resveratrol showed a dose-dependent effect with >70% inhibition at the highest concentration (10 µM). Both the In silico and in vitro analysis of resveratol as a drug for the treatment of rheumatoid arthritis are giving sufficient leads to proceed for further drug development.

Acknowledgements

The protocols/experiments involving the use of human specimens were duly examined and approved by the Hospital Ethics Committee (HEC), ZhuJiang hospital of Southern Medical University, Guangzhou (HEC-ZHSMU/2014).

Disclosure of conflict of interest

None.

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References


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