The Mechanism and Potential Targets of Betulinic Acid in the Treatment of Breast Cancer Using Network Pharmacology and Molecular Docking

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Abstract

Betulinic acid (BA) is a natural pentacyclic triterpene in many Chinese herbs, which has effectively anti-tumor biological property. Objective. To explore the anti-breast cancer (BC) mechanism and potential target proteins of BA by using network pharmacology and molecular docking. Methods. Firstly, potential BC-related target proteins of BA were screened by using Super-PRED and DisGeNET databases, and the “BA - potential target proteins against BC” network was summarized by using Cytoscape software. Then, DAVIDA database was used to perform GO and KEGG enrichment analysis to find BC-related targets in core pathways, biological process, cellular components, and the protein-protein interaction (PPI) between target proteins was analyzed by STRING database and Cytoscape software. Finally, the docking analysis of BA to BC-related targets was verified by Ledock software. Results. The results showed that there were 51 BC-related targets to BA. These targets were further mapped to 99 GO biological process, 16 GO cellular component, 17 GO molecular function, and 93 remarkable pathways. In the PPI network analysis and molecular docking verification, 5 key targets were found, including ESR1, STAT3, HSP90AA1, SIRT1, and mTOR. Conclusion. Based on the network pharmacology analysis, it is speculated that BA may not only participate in multiple mechanisms of action on treat breast cancer but may also be involved in worth of BA in clinical application of breast cancer.

Keywords: Betulinic Acid; Breast Cancer; Network Pharmacology
**Introduction**

Breast cancer (BC) is one of the most common cancers among women in the world. Over 2 million women are diagnosed with breast cancer every year throughout the world [1]. Despite high sensitivity towards surgery, chemotherapy and radiation therapy, but many patients are not benefit from these treatments because of chemo-resistance and poor-prognosis [2]. Hence, it is critical for finding the novel agents to improve the survival rate of BC patients.

Betulinic acid (BA) is a pentacyclic triterpene with a lupine structure, found in birch bark, acuminastissima leaves (Fig. 1A). Many studies have demonstrated that BA has many different biological properties: anti-inflammatory, anti-oxidative, anti-tumor, and so on [3]. Subsequent studies found that BA inhibited proliferation, arrested cell cycle, and induced apoptosis in multiple cancers, including breast cancer, non-small cell lung cancer [4, 5]. However, the underlying molecular pharmacology and potential targets of BA have not been well known.

In this study, we analyzed the potential targets of BA in the treatment of BC. Then, these targets were used to research the target pathways by network pharmacology approach, gene ontology (GO), biological pathway (KEGG) functional enrichment, and protein–protein interaction (PPI) network. Finally, molecular docking technology was used to analyze the BA dock with potential targets to explore the most binding model. This research was carried out to provide a theoretical basis for the molecular mechanisms of BA against BC.

**Materials and Methods**

**Predicting targets of BA**

To obtain the putative targets of BA, the Super-PRED (https://prediction.charite.de/subpages/target_prediction.php) databases was used. Then, the name of these identified candidate targets was inputted into UniProt database (www.Uniport.org) for normalization.

**Screening targets related to BC**

BC-related target genes were collected from DisGeNET database (https://www.disgenet.org/) and Comparative Toxicogenomics database (CTD, http://ctdbase.org/). Potential target genes (overlapping genes) of BA for BC treatment were acquired through the Veeny 2.1 (https://bioinfogp.cnb.csic.es/tools/venny/) intersection.

“BA- BC related genes” network construction

The complex relationship between BA, and BC related genes were visualized using Cytoscape software.

**GO and KEGG pathway enrichment analysis**

To investigate the effect of BA in treatment BC, the DAVID database was carried out for GO and KEGG pathway enrichment analysis. The enriched GO terms and pathways having a corrected P value of less than 0.01 were selected and subjected to further analyses.

**Protein-Protein interaction (PPI) network analysis**

The PPI network for the predicted targets of BA was constructed using the STRING database (https://string-db.org). The cut-off criterion of minimum required interaction score was set as ≥0.4 and the other parameters were default. Then, PPI network was visualized by Cytoscape.
Binding capacity between BA and BC-related targets by Molecular Docking

Ledock was used to perform molecular docking analysis on BA and the potential targets to predict their interaction activity. Firstly, the 3D structure of BA (compound CID: 64971) was obtained from PubChem (https://pubchem.ncbi.nlm.nih.gov/), and the mol2 format of 3D structure of BA was prepared in the Open Babel software. Secondly, the crystal structures of STAT3 (PDB ID: 6NUQ), HSP90AA1 (PDB ID: 5H22), mTOR (PDB ID: 4JT6), ESR1 (PDB ID: 4XI3), and SIRT1 (PDB ID: 4I5I) were obtained from the Protein Data Bank (www.rcsb.org). Then, molecular docking was performed by LeDock. All parameters were set as default. PyMol was used to display the molecular docking results.

Results

BC-related target of BA prediction and analysis

159 target genes of BA were predicted from Super-PRED database (Supplementary file 1). By means of the DisGeNET and CTD database, we obtained 1402 BC-related targets (Supplementary file 2). From an intersection from the two categories of targets, 51 overlapping target genes were found as the potential targets of BA in BC treatment (Fig 1B). As shown in Fig. 1C, the “BA-BC related targets” network was constructed, the interactions between them have 52 nodes and 51 edges.

GO and KEGG analysis of BC-related targets of BA

The results of GO analysis of 51 potential BC-related targets of BA were shown that the inflammatory response, positive regulation of angiogenesis, phosphorylation, and negative regulation of apoptotic process were significantly associated with biological processes; cellular component focuses on response to nucleoplasm, condensed chromosome, cytoplasm, membrane raft, and transcription factor complex; molecular function was mainly concentrated in kinase activity, ATP binding, 1-phosphatidylinositol-4-phosphate 3-kinase activity, protein tyrosine kinase activity, and histone deacetylase binding (Fig. 2A).
In Fig. 2B, the KEGG enrichment analysis demonstrated that the KEGG pathway mainly manifested in Thyroid hormone signaling pathway, FoxO signaling pathway, HIF-1 signaling pathway, PI3K-AKT signaling pathway, and AMPK signaling pathway. Taken together, these results of GO and KEGG pathway enrichments suggested that 51 predicted BC-related targets had the key role in BA treatment for BC.

**PPI network of BC-related targets of BA**

PPI network of 51 potential BC-related targets of BA was constructed using STRING database. As shown in Fig. 3, the top targets with the high degree (the number of lines linked to a given node) were HSP90AA1, STAT3, ESR1, SIRT1, mTOR.
Molecular docking analysis

To verify the reliability of BA and BC-related targets, HSP90AA1, STAT3, ESR1, SIRT1, mTOR were selected as receptor for molecular docking based on the above results. The docking scores for each target, shown in Table 2, suggested that there was a strong interaction between BA and the 5 targets.

<table>
<thead>
<tr>
<th>NO.</th>
<th>Target Name</th>
<th>Gene Symbol</th>
<th>NO.</th>
<th>Target Name</th>
<th>Gene Symbol</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Bloom syndrome protein</td>
<td>BLM</td>
<td>27</td>
<td>Sodium/hydrogen exchanger 1</td>
<td>SLC9A1</td>
</tr>
<tr>
<td>2</td>
<td>Nuclear factor NF-kappa-B p105 subunit</td>
<td>NFKB1</td>
<td>28</td>
<td>Protein tyrosine kinase 2 beta</td>
<td>PTK2B</td>
</tr>
<tr>
<td>3</td>
<td>Cathepsin D</td>
<td>CTSD</td>
<td>29</td>
<td>Histone deacetylase 9</td>
<td>HDAC9</td>
</tr>
<tr>
<td>4</td>
<td>Indoleamine 2,3-dioxygenase</td>
<td>IDO1</td>
<td>30</td>
<td>AMP-activated protein kinase, alpha-1 subunit</td>
<td>PRKAA1</td>
</tr>
<tr>
<td>5</td>
<td>Cytochrome P450 3A4</td>
<td>CYP3A4</td>
<td>31</td>
<td>Serine/threonine-protein kinase mTOR</td>
<td>MTOR</td>
</tr>
<tr>
<td>6</td>
<td>LSD1/CoREST complex</td>
<td>KDM1A</td>
<td>32</td>
<td>PI3-kinase p110-beta subunit</td>
<td>PIK3CB</td>
</tr>
<tr>
<td>7</td>
<td>Signal transducer and activator of transcription 3</td>
<td>STAT3</td>
<td>33</td>
<td>Bcr/Abl fusion protein</td>
<td>ABL1</td>
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<tr>
<td>8</td>
<td>MAP kinase ERK2</td>
<td>MAPK1</td>
<td>34</td>
<td>C-C chemokine receptor type 2</td>
<td>CCR2</td>
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<tr>
<td>9</td>
<td>Cyclin-dependent kinase 1/cyclin B1</td>
<td>CDK1</td>
<td>35</td>
<td>Cyclooxygenase-1</td>
<td>PTGS1</td>
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<tr>
<td>10</td>
<td>Cannabinoid CB2 receptor</td>
<td>CNR2</td>
<td>36</td>
<td>Cyclin-dependent kinase 2/cyclin A</td>
<td>CDK2</td>
</tr>
<tr>
<td>11</td>
<td>PI3-kinase p110-alpha/p85-alpha</td>
<td>PIK3R1</td>
<td>37</td>
<td>B-cell lymphoma 6 protein</td>
<td>BCL6</td>
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<td>12</td>
<td>ADAM10</td>
<td>ADAM10</td>
<td>38</td>
<td>3-phosphoinositide dependent protein kinase-1</td>
<td>PDPK1</td>
</tr>
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<td>13</td>
<td>Toll-like receptor 4</td>
<td>TLR4</td>
<td>39</td>
<td>NAD-dependent deacetylase sirtuin 1</td>
<td>SIRT1</td>
</tr>
<tr>
<td>14</td>
<td>DNA-(apurinic or apyrimidinic site) lyase</td>
<td>APEX1</td>
<td>40</td>
<td>Estrogen receptor alpha</td>
<td>ESR1</td>
</tr>
<tr>
<td>15</td>
<td>Androgen Receptor</td>
<td>AR</td>
<td>41</td>
<td>Sphingosine kinase 1</td>
<td>SPHK1</td>
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<td>16</td>
<td>Cyclin-dependent kinase 4/cyclin D1</td>
<td>CDK4</td>
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<td>PI3-kinase p110-gamma subunit</td>
<td>PIK3CG</td>
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<tr>
<td>17</td>
<td>Kruppel-like factor 5</td>
<td>KLF5</td>
<td>43</td>
<td>Bromodomain-containing protein 4</td>
<td>BRD4</td>
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<tr>
<td>18</td>
<td>DNA topoisomerase II alpha</td>
<td>TOP2A</td>
<td>44</td>
<td>Telomerase reverse transcriptase</td>
<td>TERT</td>
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As showed in Fig. 4, Leu346, Thr347, Ala350, Asp351, located in functional domain, were the amino acid residues in the binding sites of BA to ESR1 (Fig. 4A); while Asn-51, Asp-54, Ile-91, Gly-97, Met-98, and Leu-107 were responsible for BA binding to HSP90AA1 (Fig. 4B); For analyzing the binding sites between BA and mTOR, Ser-2165, Gln-2167, Pro-2169, Trp-2239, Val-2240, Cys-2243 were involved in stabilizing binding poses (Fig. 4C); The amino acid residue of SIRT1, interacted with BA were Phe-297, Ile-316, Phe-413, Phe-414, Gly-440, and Ser-441 (Fig. 4D); The bonds between BA and residues of STAT3 (Tyr-640, Gln-644, and Met-648) may be vital in BA binding to STAT3, the worth of Ser-613, Trp-623, Val-637, Glu-638, Asn-647, and Tyr-657 could not be ignored (Fig. 4E).

Table 1: The potential targets of BA against breast cancer

<table>
<thead>
<tr>
<th>NO.</th>
<th>Target Name</th>
<th>Gene Symbol</th>
<th>NO.</th>
<th>Target Name</th>
<th>Gene Symbol</th>
</tr>
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<tr>
<td>19</td>
<td>P-glycoprotein 1</td>
<td>ABCB1</td>
<td>45</td>
<td>Matrix metalloproteinase 8</td>
<td>MMP8</td>
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<tr>
<td>20</td>
<td>Acyl-CoA desaturase</td>
<td>SCD</td>
<td>46</td>
<td>PI3-kinase p110-delta subunit</td>
<td>PIK3CD</td>
</tr>
<tr>
<td>21</td>
<td>Nerve growth factor receptor Trk-A</td>
<td>NTRK1</td>
<td>47</td>
<td>Heat shock protein HSP 90-alpha</td>
<td>HSP90AA1</td>
</tr>
<tr>
<td>22</td>
<td>UDP-glucuronosyl transferase 2B7</td>
<td>UGT2B7</td>
<td>48</td>
<td>Cyclin-dependent kinase 2/cyclin E1</td>
<td>CCNE1</td>
</tr>
<tr>
<td>23</td>
<td>Proteasome subunit beta type-9</td>
<td>PSMB9</td>
<td>49</td>
<td>Matrix metalloproteinase 7</td>
<td>MMP1</td>
</tr>
<tr>
<td>24</td>
<td>Retinoid X receptor beta</td>
<td>RXRB</td>
<td>50</td>
<td>Glucose transporter SLC2A1</td>
<td>SLC2A1</td>
</tr>
<tr>
<td>25</td>
<td>Heparanase</td>
<td>HPSE</td>
<td>51</td>
<td>Cathepsin B</td>
<td>CTSB</td>
</tr>
<tr>
<td>26</td>
<td>EP300</td>
<td></td>
<td></td>
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Table 2: Molecular docking parameters and results of five BC-related targets binding with BA

<table>
<thead>
<tr>
<th>PDB ID</th>
<th>Protein Name</th>
<th>Scores (kcal/mol)</th>
<th>Ledock pocket center</th>
<th>x</th>
<th>y</th>
<th>z</th>
</tr>
</thead>
<tbody>
<tr>
<td>5H22</td>
<td>HSP90AA1</td>
<td>-5.01</td>
<td>-39.638, 22.275</td>
<td>-5.01</td>
<td>22.75</td>
<td></td>
</tr>
<tr>
<td>4JT6</td>
<td>mTOR</td>
<td>-5.28</td>
<td>-39.165, 27.439</td>
<td>-6.943</td>
<td>47.536</td>
<td></td>
</tr>
<tr>
<td>4XI3</td>
<td>ESR1</td>
<td>-4.51</td>
<td>-27.128, 8.585</td>
<td>-4.51</td>
<td>8.585</td>
<td></td>
</tr>
<tr>
<td>4I5I</td>
<td>SIRT1</td>
<td>-4.48</td>
<td>3.7, 23.5</td>
<td>-4.48</td>
<td>23.5</td>
<td></td>
</tr>
<tr>
<td>6NUQ</td>
<td>STAT3</td>
<td>-4.31</td>
<td>3.7, 23.5</td>
<td>-4.31</td>
<td>23.5</td>
<td></td>
</tr>
</tbody>
</table>

Figure 4: Molecular docking of BA with the core BC-related targets. (A) Molecular docking of BA with ESR1. (B) Molecular docking of BA with mTOR. (C) Molecular docking of BA with HSP90AA1. (D) Molecular docking of BA with SIRT1. (E) Molecular docking of BA with STAT3.
Discussion

The network pharmacology has been significantly promoted an understanding of the complex interactions between drugs and their targets and the potential mechanisms of action. It is usually applied for studying the novel targets of Traditional Chinese Medicine, and drug repurposing [6-8]. In this work, we explored the potential mechanism and targets of BA against BC by using reverse-docking analysis, GO, KEGG enrichment analysis, and PPI analysis. The results demonstrated that 5 targets including HSP90AA1, ESR1, SIRT1, mTOR, and STAT3 which have a well combination with BA were selected as potential targets for BA in BC therapy.

Previous studies have revealed that BA display anti-tumor biological effects in BC cells. Jiao et al. found that BA inhibited aerobic glycolysis activity for reducing proliferation via caveolin-1/NF-kB/c-Myc pathway in BC cell lines MCF-7 and MDA-MB-231[9]. Zheng et al. suggested that BA could attenuate migration and invasion of highly aggressive breast cancer cells by targeting GRP78 mediated glycolysis and ER stress apoptotic pathway [10]. In addition, BA increases expression of p53 and p21, which are in cell cycle regulation, and arrest MCF-7 in G1 phase [11]. BA mediated inhibition of topoisomerase I and II in MDA-MB-231 cells induces cell cycle arrest and apoptosis [12, 13]. Other research found that BA down-regulates sp1, ROS, and VEGFR pathways [14-16].

In this study, we predicted 149 pharmacophore candidates via Supra- and found 51 potential targets of BA against BC. Moreover, analysis of GO and KEGG enrichment results for 51 targets suggested that 134 biological functions and 98 signaling pathways are directly involved in the occurrence and development of BC, suggesting that these gene function and signaling pathways may be the mechanism by which BA can treat BC. Subsequently, the results of PPI analysis and molecular verification demonstrated that BA could directly bind to HSP90AA1, ESR1, SIRT1, mTOR, and STAT3 for regulating thyroid hormone, FoxO, HIF-1, PI3K-AKT, AMPK signaling pathway.

Heat shock protein 90-alpha (HSP90AA1), an essential molecular chaperon that is highly conserved in evolution, is efficiently expressed under the stimulatory conditions of trauma, infection, and tumors [17-19]. In breast cancer, HSP90AA1 is required for the stabilization of many proteins in pathways that play key roles in cancer growth and survival, such as estrogen receptor (ER), progesterone receptor (PR), essential components of HER2 signaling (HER2, AKT, c-SRC, RAF and HIF-1α), and EGFR [20, 21]. Hence, HSP90AA1 has been acted as a candidate marker for diagnosis, prognosis, and therapy of BC.

Estrogen receptor alpha gene (ESR1) regulates the expression of estrogen receptors, involved in the regulation of gene expression, and affect proliferation and differentiation in target tissues [22]. In BC cells, it has been suggested that ESR1 can mediates PI3K-AKT signaling pathway to promote cell growth [23].

Numerous contributions support the notion that SIRT1, a type III histone deacetylase, activities influence hormone receptors (HR) actions, expressly those mediating long-term estrogenic effects in the mammary gland, namely the classic ERs, for which a relevant degree of interdependence between these factors has been described with apparent importance for BC onset and development. Moreover, SIRT1 shows a convoluted role in the regulation of the epithelial-mesenchymal transition (EMT) process, with apparent relevance in the case of reproductive tumors such as BC [24]. Moreover, Xu et al found that FoxO family members were recruits into active ERα and SIRT1 transcriptomic complexes [25]. Additionally, SIRT1 up-regulates PI3K-AKT, MEK-ERK, and GPER pathway [26,27].

Signal transducer and activator of transcription 3 (STAT3), phosphorylated by IL-6 and JAK2 at Tyr 705, has been shown to play an important role in BA cells by inducing EMT and angiogenesis [28]. VEGFR, PKC, PI3K also could mediate constitutive STAT3 activation [29-31].

In mammalian cells, it exists as two distinct multiprotein complexes: mTORC1 and mTORC2. mTORC1 is sensitive to Rapamycin and promotes cell growth and survival by stimulating nutrient uptake and metabolism. It also stimulates cell growth by promoting ribosome production and protein synthesis and by inhibiting protein degradation [32]. mTORC1 may be activated through differ-
ent pathways, but mainly through the PI3P/AKT pathway, which is activated by extracellular growth factors and nutrients [33]. Inhibitors of mTOR such as rapamycin (sirolimus) and the rapalogs temsirolimus (CCI-779), everolimus (RAD001), and deforolimus (AP23573) have been extensively evaluated in ER-positive, and HER2-overexpressing breast cancer, hematological malignancies, renal cancer, and as treatment for transplant rejection [34]. The results of molecular docking analysis, suggested that BA could bind the functional domain of these targets, and validated the reliability of interaction between BA and BC related targets screened by network pharmacology.

In summary, this study used a network pharmacology approach to construct a network to display the interactions between BA and BC-related target genes. The results indicate that BA may exert anti-breast cancer effect by acting on SIRT1, ESR1, STAT3, HSP90AA1, and mTOR, and also speculated that BA may participate in multiple mechanisms of action to treat breast cancer. Therefore, it is considered that BA can be a candidate drug in the anti-breast cancer therapy.

**Acknowledgements**

The present study was supported by the National Science Foundation of China (Grant Nos. 81502958), the National natural Science Foundation of Jiangsu Province (Grant Nos. BK20150286,) and Minsheng Science and Technology Project of Suzhou (Grant Nos. SS202086, SKJY2021032).
References


