RESEARCH ARTICLE

Exploring the underlying mechanism of action of a traditional Chinese medicine formula, Youdujing ointment, for cervical cancer treatment

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Background: A traditional Chinese medicine formula, Youdujing (YDJ) ointment, is widely used for treatment of human papilloma virus-related diseases, such as cervical cancer. However, the underlying mechanisms by which active compounds of YDJ alleviates cervical cancer are still unclear.

Methods: We applied a comprehensive network pharmacology approach to explore the key mechanisms of YDJ by integrating potential target identification, network analysis, and enrichment analysis into classical molecular docking procedures. First, we used network and enrichment analyses to identify potential therapeutic targets. Second, we performed molecular docking to investigate the potential active compounds of YDJ. Finally, we carried out a network-based analysis to unravel potentially effective drug combinations.

Results: Network analysis yielded four potential therapeutic targets: ESR1, NFKB1, TNF, and AKT1. Molecular docking highlighted that these proteins may interact with four potential active compounds of YDJ: E4, Y2, Y20, and Y21. Finally, we found that Y2 or Y21 can act alone or together with E4 to trigger apoptotic cascades via the mitochondrial apoptotic pathway and estrogen receptors.

Conclusion: Our study not only explained why YDJ is effective for cervical cancer treatment, but also lays a strong foundation for future clinical studies based on this traditional medicine.

Keywords: Youdujing; cervical cancer; traditional Chinese medicine; network pharmacological; molecular docking; synergy effect

Author summary: The mechanisms underlying the effect of many traditional Chinese medicine remained unclear, so we developed a network pharmacology method to investigate the active compounds and their possible combinations by integrating network and enrichment analyses with molecular docking. In this paper, we found four potential active compounds and four potential therapeutic targets of YDJ. However, these findings should be confirmed by further experiments in vitro and in vivo, whose results can be integrated in the present bioinformatic algorithm in order to optimize our method in the future.

INTRODUCTION

Cervical cancer (CC) is one of the most common malignant tumors afflicting women [1,2]. Indeed, it has been estimated that in 2018 approximately 570,000 new cases and 310,000 deaths were due to this disease worldwide [3]. In particular, the incidence of CC in developing countries is much higher than in developed
countries because of inadequate screening [3]. Although preventive vaccines against human papilloma virus (HPV) can decrease the incidence and mortality of CC [4], these are neither effective for infected patients [5] nor affordable for patients in developing countries [6]. Therefore, drug-based treatments are becoming the most common method to cure HPV-infected patients in developing countries [7–9]. Although platinum-based chemotherapy has been used to treat CC for years, the survival rate of CC patients did not improve significantly until bevacizumab was developed [1]. However, bevacizumab cannot be considered an ideal drug because of its high price [10], side effects [11] and incomplete effectiveness [12]. For this reason, it is urgent to find an effective, low-cost, and side effect-free treatment for CC.

Recently, clinical use of traditional Chinese medicine (TCM) formula Youdujing (YDJ) was reported to treat multiple diseases including HPV-dependent CC [13,14] because of its moderate therapeutic effects and lesser side effects [15,16]. Interestingly, Xiao et al. [17] found that YDJ can counteract HPV infection and lead to an almost complete recovery of local lesions. However, the mechanisms underlying the effect of YDJ remained unclear due to the following reasons. First, the etiology of CC is so complex that it is difficult to identify the most effective therapeutic targets. Second, since this TCM formula includes hundreds of chemical compounds acting on multiple targets, it is difficult to pinpoint its active compounds and localize the sites where these exert their effects. Third, because there are too many drug combinatorial possibilities [18,19], it is difficult to explore optimal drug combinations exhibiting the best clinical efficacy and the least side effects.

To address these issues, we developed a network pharmacology method to investigate the active compounds of YDJ ointment and their possible combinations by integrating network [18,20] and enrichment analyses [21–29] with molecular docking [19,30]. First, we identified potential therapeutic targets using network and enrichment analyses to explore hub nodes and their related biological processes. Second, we utilized molecular docking to compute the binding energies between the chemical compounds of YDJ and their potential therapeutic targets to predict the active compounds of YDJ. Finally, we used network-based proximity parameters to investigate the optimum drug combinations.

We obtained the following interesting findings. First, network and enrichment analyses yielded four potential therapeutic targets: ESR1, NFKB1, TNF, and AKT1, which were confirmed by manual review of existing literature [31–34]. Second, molecular docking allowed to identify four potential active compounds: E4, Y2, Y20, and Y21. Interestingly, E4, Y2, and Y21 were shown to promote apoptosis [35–37]. Third, after using network-based proximity to estimate the relationships between E4, Y2, and Y21, we considered Y2-E4 and Y21-E4 as potential drug combinations and demonstrated that Y2 or Y21 can act together with E4 to trigger apoptotic cascades via the mitochondrial apoptotic pathway [38] and/or estrogen receptors [39].

In general, this study not only describes the reason why YDJ is effective for CC treatment, but also offers a general procedure to investigate the underlying mechanism of action of TCM formulas.

RESULTS

Step 1: Data collection

The YDJ ointment consists of three herbs, *Bracea javanica* (L.) Merr., *Curcuma phaeocaulis* val., and *Arnebia euchroma* [14], from which we collected 30, 21, and 10 active chemical compounds, respectively, as illustrated in step 1 of Fig. 1. MedChem Studio [40] was used to identify the potential targets of these compounds. After examining duplicate information and removing redundancies, we obtained 320 potential targets, which are detailed in Supplementary Tables S1–S3. Next, we downloaded the sequences of 5003 proteins known to be targeted during CC treatment from the GeneCard database [41]. Finally, we intersected the 320 YDJ potential targets with the 5003 CC-related proteins to obtain 119 candidate targets (Supplementary File 1) at the end of step 1.

Step 2: Identify potential key targets of YDJ involved in cervical cancer

In step 2, network and enrichment analyses were employed to identify key targets of YDJ involved in CC to answer our first scientific question: “How to identify the most effective therapeutic targets for the complicated etiology of CC”. We first used two topological features (degree and BC) to identify hub nodes in the protein-protein interaction (PPI) network; then, we carried out enrichment analysis to identify genes involved in important Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways. Finally, we intersected these results to pinpoint potential key targets.

Using network analysis to predict hub nodes

First, we input the above-mentioned 119 putative targets (step 2 of Fig. 1) into the STRING database to compute PPI scores, whose threshold was set to 0.9 (the highest confidence). Disconnected nodes were hidden. The generated PPI network included 90 nodes and 203 edges. Next, we examined the topological features illustrated in the section of Materials and Methods (i.e.,
Figure 1. Network pharmacology procedure to investigate the underlying mechanism of action of YDJ.
degree and betweenness centrality, BC) to identify the potential key targets of YDJ (Fig. 2). We obtained 10 hub nodes (Table 1) regarding to the thresholds of degree and BC (Supplementary Table S4). In addition, Fig. 2 shows that the average node degree was 3.41, and the node degree distribution in the PPI network followed a power law with a long tail.

Using enrichment analysis to identify targets

To understand the pharmacological mechanisms of the effect of YDJ on CC, we carried out enrichment analysis of the proteins of the PPI network depicted in Fig. 2 using DAVID v6.8 [42]. Supplementary Table S4 indicates the threshold of false discovery rates (FDR) applied for Gene Ontology (GO) and KEGG enrichment analysis. These enrichment analyses yielded 140 GO biological processes and 73 KEGG pathways, which are detailed in Supplementary Tables S5–S6.

Since previous epidemiological studies have demonstrated that HPV infection [43], smoking [44] and oral contraceptive use [45] are important causes of CC, we considered that drugs acting on these pathways and their corresponding receptors could be effective for CC treatment [46]. Thus, we highlighted GO biological processes (Table 2) and KEGG pathways (Table 3) related to HPV infection [43], smoking [44] and oral contraceptive use [45] from Supplementary Tables S5–S6.

Table 2 shows the GO biological processes predicted to be most related to CC: regulation of reactive oxygen species metabolic process (GO0048545) and response to steroid hormone (GO0048545). Table 3 shows the KEGG pathways predicted to be most related to CC: oxidative phosphorylation (hsa00190), human papillomavirus infection (hsa05165), estrogen signaling pathway (hsa04915), and steroid hormone biosynthesis (hsa00140).

Finally, we intersected the results of network analysis (Table 1) and enrichment analysis (Table 3) to obtain the four key targets listed in Table 4.

**Step 3: Screening of potential active compounds**

To answer our second scientific question: “since this TCM formula includes hundreds of chemical compounds acting on multiple targets, how to pinpoint its active compounds and localize the sites where these exert their effects”, we followed the procedure of Chen et al. [51] as illustrated in
step 3 of Fig. 1. First, we calculated the binding energy threshold ($\Delta G_{i,j}$) between targets $i$ ($i = \text{ESR1, TNF, AKT1, and NFKB1}$) and their intrinsic ligands $j$ ($j = \text{XBR, 307, XM1 and Compound 5}$) by Autodock Vina [30]. The results are listed in Supplementary Table S7.

Second, we calculated the binding energy ($\Delta G_{i,j}$) between targets $i$ and their ligands $j$ (“Ligands” in Table 4) using Autodock Vina [30]. Then, we identified the active compounds with strong binding energies (Fig. 3) by comparing the binding energy of each target-ligand pair ($\Delta G_{i,j}$) with their corresponding thresholds ($\Delta G_{i,j}$).

As shown in Fig. 3A, we determined that seven compounds could bind to ESR1, whereas only compounds Y20 and E4 displayed lower binding energy than the intrinsic ligand XBR [47, 48]. Moreover, three compounds could bind to TNF, whereas only compounds Y2 and Y21 exhibited lower binding energy than the intrinsic ligand 307 (Fig. 3B). Conversely, only one compound, E4, could bind to AKT1, but this showed a greater binding energy than the intrinsic ligand XM1 (Fig. 3C). Finally, four compounds could bind to NFKB1, although only compound Y2 displayed lower binding energy than the intrinsic ligand Compound 5 (Fig. 3D). Therefore, we believe that Y2, Y20, Y21, and E4 could be potential active compounds of YDJ (Table 5).

**Step 4: Predict potential drug combinations**

To answer our third scientific question: “because there are too many drug combinatorial possibilities [18,19], how to explore optimal drug combinations exhibiting the best clinical efficacy and the least side effects”, we used a network-based method [18] to identify effective drug combinations, as illustrated in step 4 of Fig. 1. First, we determined the potential target disease module based on the function of the above-mentioned compounds (Table 5). Y2, Y21, and E4 were found to be related to apoptosis, while the function of Y20 was unclear. Thus, we removed Y20 from the list of potential active compounds and considered the apoptotic process as the disease module of interest. Second, we computed network-based proximity ($S_{AB}$) to measure the relationships...
between Y2, E4, and Y21. $S_{Y2-Y21}$, $S_{Y2-E4}$, and $S_{Y21-E4}$ were found to equal $-0.207$, $0.333$, and $0.200$, respectively.

As described in the section of Materials and Methods, a $S_{Y2-Y21} < 0$ indicated that the targets of Y2 and Y21 were adjacent to each other in the PPI network (Fig. 4A). A close network proximity of two targets indicates highly similar biological functions. Conversely, E4 targets were separated from those of Y2 or Y21 (Fig. 4B,C); consequently, E4 and Y2 (or Y21) were predicted to be pharmacologically different. Only in this case, compound pairs show statistically significant efficacy during combinatorial therapies [18, 56, 57]. Therefore, we selected the drug pairs characterized by $S_{AB} > 0$ and considered Y2-E4 and Y21-E4 as effective drug combinations.

DISCUSSION

This research aimed to develop a network pharmacology framework (Fig. 1) to investigate the active compounds of YDJ and their possible combinations for CC treatment. First, we collected available data on CC-related proteins (Supplementary File 1), chemical compounds of YDJ, and their corresponding targets (Supplementary Tables S1–S3).

Second, we identified four potential key targets: ESR1, TNF, AKT1, and NFKB1 by intersecting the results of network analysis (Table 1) and enrichment analysis (Tables 2 and 3). ESR1 was found to be closely associated with CC [58]. As CC can depend on excessive estrogen stimulation, drugs acting on ESR1 could contribute to the treatment and/or prevention of CC [59]. Moreover, James et al. showed that the E6 protein of HPV16 can inhibit TNF-induced apoptosis, thereby contributing to cell immortalization and carcinogenesis [32]. Therefore, the stimulation of TNF-induced apoptosis could have a great impact on the treatment of CC [60, 61]. Furthermore, Cui et al. demonstrated that SLUG can suppress the
proliferation of CC cells by inhibiting the expression of AKT1 [34]. Finally, NFKB1 plays an important role in inflammation, which can induce accumulation of reactive oxygen species (ROS), thereby leading to an increased risk of CC [33]. In recent years, many studies have demonstrated that natural antioxidants can regulate cellular signal transduction pathways through the activation or inhibition of NFKB1 [33,62,63]. Since these four targets corresponded to the hub nodes in the PPI network (Table 1), we considered them to be potential therapeutic targets.

Third, we analyzed the relationships between the four targets and their corresponding ligands by molecular docking (Table 4) and identified four active compounds of YDJ: Y2, Y20 Y21, and E4 (Fig. 3 and Supplementary Table S7). Y2, Y21, and E4 were found to be related to apoptosis, while the function of Y20 was unclear (Table 5). E4 is a dihydroxyflavanone with versatile pharmacological activities. For example, many studies have demonstrated that E4 can inhibit cancer cell growth by inducing apoptosis [35,55]. In addition, Zhao et al. [64] proved that E4 can interact with the human estrogen receptors ESR1 and ESR2. Y21 is a triterpene, reported to significantly inhibit cancer cell proliferation and promote apoptosis [37,54]. Finally, Y2 is a steroid saponin, which has been demonstrated to be effective for inducing apoptosis [36,52,53].

Fourth, we used a network-based method to identify two potential drug combinations: Y2-E4 and Y21-E4 (Fig. 4). We predicted that E4 can act together with both Y21 and Y2 to trigger cell apoptosis through mitochondrial apoptotic pathways and estrogen receptors (Fig. 5).

Based on the principle of Sovereign-Minister-Assistant-Envoy (Jun-Chen-Zuo-Shi in Chinese) [65,66], we propose Y2 and Y21, the active compounds of B. javanica (L.) Merr., as the sovereign of the therapeutic process, since they can inhibit NFKB1 and promote TNF-induced apoptosis [60,61]. In addition, we consider E4, the active compound of C. phaeocaulis val., as a minister acting in synergy for the induction of apoptosis. Indeed, E4 can generate increased ROS accumulation to promote the activity of tumor suppressor proteins, thereby triggering apoptotic cascades through mitochondrial apoptotic pathways (Fig. 5) [38,67]. Moreover, E4 may trigger apoptotic cascades via estrogen receptors. In fact, E4 exhibited estrogenic activity with an EC50 value of 67 µM [64] and ESR1 can initiate estrogen-triggered apoptotic pathways [39]. Therefore, we conclude that E4 is a phytoestrogen that can compete with estrogen for binding to ESR1 to promote the activation of apoptotic pathways (Fig. 5).

Here we investigated the potential targets, active compounds, and drug combinations enabling YDJ-mediated CC treatment using a network pharmacology approach. Four proteins related to apoptosis (ESR1, TNF, AKT1, and NFKB1) were highlighted as potential therapeutic targets. Moreover, the compounds E4, Y21, and Y2 exhibited greater binding energies for ESR1, TNF, and NFKB1, respectively, than the respective thresholds. Finally, Y2-E4 and Y21-E4 were denoted as potentially effective drug combinations. Indeed, Y2 (or Y21) can act together with E4 to trigger apoptotic cascades via the
mitochondrial apoptotic pathway and/or estrogen receptors. However, these findings should be confirmed by further experiments in vitro and in vivo, whose results can be integrated in the present bioinformatic algorithm [26–29,68] in order to optimize our method in the future.

**MATERIALS AND METHODS**

We developed a network pharmacology method to investigate the active compounds of YDJ ointment and their possible combinations, as shown in Fig. 1. In this process, step 1 was to collect information on the active compounds of YDJ and their CC-related targets from public databases. Then, step 2 was to pinpoint hub genes through network analysis. Next, step 3 was to investigate the related biological processes and signaling pathways by enrichment analysis. Lastly, step 4 was to identify the active compounds by molecular docking and to estimate compound-compound relationships using a network-based approach.

**Step 1: Data collection**

Predict YDJ potential targets

The identities of the chemical compounds of YDJ ointment were collected from the TCMIP database [69]. To assess the similarity between these compounds and the respective associated drugs (Fig. 1), we calculated the molecular fingerprint [70] of each compound or drug using RDkit, and then we measured the similarity between the compound and the drug using the Tanimoto score in Eq. (1) [71]. The example of fingerprint is listed in Supplementary File 3. Here, we denote fingerprint as a sequence of bits transformed from the molecular.

The Tanimoto score in Eq. (1), an index of the structural similarity between the chemical compounds of YDJ and known drugs, was used to identify active compounds.

\[
\text{Tanimoto score}(a,b) = \frac{c}{a + b - c} \tag{1}
\]

where \(a\) equals the amount of bit set to 1 in the molecular fingerprint of the YDJ compound, \(b\) is the amount of bit set to 1 in the molecular fingerprint of the known drug in DrugBank [72], and \(c\) equals the amount of bit set to 1 in both. Herein, according to the default threshold used by TCMIP [69], we set the Tanimoto score threshold to 0.8. Compounds were considered active, if Tanimoto score was greater than the threshold.

Proteins related to cervical cancer

The candidate target proteins for treatment of CC were obtained from the GeneCard database [41].

**Step 2: Identify potential key targets**

Protein-protein interaction network and network analysis

First, we intersected the list of potential targets of YDJ and that of CC-related proteins. Second, we used this intersection to construct a PPI network by STRING (version 11.0) [73]. PPI scores were calculated using Eq. (2).
where $x$ and $y$ represent the gene expression values of different genes, respectively; $\bar{x}$ and $\bar{y}$ represent the mean values of $x$ and $y$, respectively; and $n$ is the number of experiments. The threshold value of PPI scores was set to 0.9 (the highest confidence). Disconnected nodes were hidden in the PPI network visualized by Cytoscape (version 3.7.2) [74]. Most PPI networks are scale free [75], that is, they are based on a small number of hub proteins (hubs are nodes with high degrees). Hubs represent the most vulnerable points of disease-related protein networks [76], and thus are considered potential therapeutic targets [75].

However, since Lamb et al. demonstrated that the degree might not be the only influential parameter for the identification of crucial proteins from PPI networks [77], we considered BC, described by Eq. (3), as another important parameter for pinpointing therapeutic targets [78,79].

$$
C_i = \sum \frac{\delta_{st,i}}{\bar{\delta}_{st}},
$$

where $\delta_{st}$ represents the number of shortest paths from node $s$ to node $t$, and $\bar{\delta}_{st,i}$ indicates the number of shortest paths connecting node $s$ and node $t$ through node $i$. BC measures the importance of node $i$ in mediating the shortest path between node $s$ and node $t$. Therefore, the network topology structure was based on these two topological features.

**Step 4: Predict potential drug combinations**

We used network-based proximity Eq. (5) [18] to estimate compound–compound relationships.

$$
S_{AB} = \langle d_{AB} \rangle - \frac{\langle d_{AA} \rangle + \langle d_{BB} \rangle}{2},
$$

where $\langle d_{AA} \rangle$ and $\langle d_{BB} \rangle$ are the shortest distances within the interactome between the targets of each compound, and $\langle d_{AB} \rangle$ is the shortest distance between the A-B target pairs. The pseudocodes and workflows for the computation of $\langle d_{AA} \rangle$, $\langle d_{BB} \rangle$, and $\langle d_{AB} \rangle$ are listed in Supplementary Table S8. If $S_{AB} < 0$, the targets of compounds A and B are adjacent to each other, and a close network proximity of two targets indicates highly similar biological functions. In this case, instead of being statistically more effective than monotherapy, these compound pairs exert statistically significant adverse effects [18]. If the $S_{AB} > 0$, the targets of the two compounds are separated in the network. Therefore, two compounds are pharmacologically different. Only in this situation, these compound pairs show statistically significant efficacy for combinatory therapies [18,56,57].

**SUPPLEMENTARY MATERIALS**

The supplementary materials can be found online with this article at https://doi.org/10.15302/J-QB-021-0236.

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**COMPLIANCE WITH ETHICS GUIDELINES**

The authors Lei Zhang, Ji Lv, Ming Xiao, Li Yang and Le Zhang declare that they have no conflict of interest or financial conflicts to disclose.

All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted, and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

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Exploring the underlying mechanism of Youdujing ointment for cervical cancer

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