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Deciphering Key Pharmacological Pathways of Qingdai Acting on Chronic Myeloid Leukemia Using a Network Pharmacology-Based Strategy

Authors' Contribution:
Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
Funds Collection G

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Qingdai, a traditional Chinese medicine (TCM) used for the treatment of chronic myeloid leukemia (CML) with good efficacy, has been used in China for decades. However, due to the complexity of traditional Chinese medicinal compounds, the pharmacological mechanism of Qingdai needs further research. In this study, we investigated the pharmacological mechanisms of Qingdai in the treatment of CML using network pharmacology approaches.

First, components in Qingdai that were selected by pharmacokinetic profiles and biological activity predicted putative targets based on a combination of 2D and 3D similarity measures with known ligands. Then, an interaction network of Qingdai putative targets and known therapeutic targets for the treatment of chronic myeloid leukemia was constructed. By calculating the 4 topological features (degree, betweenness, closeness, and coreness) of each node in the network, we identified the candidate Qingdai targets according to their network topological importance. The composite compounds of Qingdai and the corresponding candidate major targets were further validated by a molecular docking simulation.

Seven components in Qingdai were selected and 32 candidate Qingdai targets were identified; these were more frequently involved in cytokine-cytokine receptor interaction, cell cycle, p53 signaling pathway, MAPK signaling pathway, and immune system-related pathways, which all play important roles in the progression of CML. Finally, the molecular docking simulation showed that 23 pairs of chemical components and candidate Qingdai targets had effective binding.

This network-based pharmacology study suggests that Qingdai acts through the regulation of candidate targets to interfere with CML and thus regulates the occurrence and development of CML.

MeSH Keywords: Leukemia, Myelogenous, Chronic, BCR-ABL Positive • Medicine, Chinese Traditional • Molecular Mechanisms of Pharmacological Action • Protein Interaction Maps

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Background

Chronic myeloid leukemia (CML) is a clonal hematopoietic stem cell proliferation-induced myeloproliferative disease [1]. It has high heterogeneity and distinct molecular genetic features – the unique cytogenetic features of CML are Philadelphia chromosome t (9; 22) (q34; q11) – in which the c-ABL protooncogene on the long arm of chromosome 9 translocates to the BCR of the long arm of chromosome 22, forming an BCR-ABL fusion gene [2,3], and it has become an important topic of research. Imatinib mesylate and the newer BCR-ABL tyrosine kinase inhibitors are the standard therapy for CML [4], which greatly improves the survival of patients with chronic myeloid leukemia; however, drug resistance and adverse effects remain a problem [5]. Therefore, looking for new strategies to improve the treatment of chronic myeloid leukemia treatment has important clinical significance.

Chinese herbal medicine is a unique medicine used in Chinese medicine to prevent and treat diseases. With the development of medicine around the world, China's ancient Chinese medicine system is receiving the attention of the world. However, it is the most important and difficult task for Chinese traditional medicine to elucidate the interaction between the complex chemical systems of traditional Chinese medicine and the complex systems of diseases and syndromes. Qingdai is prepared as clumps of dry powder, obtained by machining the leaves or stems of *Strobilanthes cusia*, *Polygonum tinctorium Ait*, and *Isatis indigotica Fort* (Pharmacopoeia of the People's Republic of China, 2010). Qingdai is one herb in Qing Huang San, which has been recorded in the "Jing Yue Quan Shu," "Shi Yi De Xiao Fang," "Qi Xiao Liang Fang," and so on, and is Professor Zhou Aixiang's classical prescription of CML treatment [6]. As confirmed by research, indirubin, a component of Qingdai, is indeed effective in the treatment of chronic myeloid leukemia [7]. Dai et al. treated K562 cells with different concentrations of Qingdai compound (2.5, 5, 7.5, 10, and 20 ug/ml) and harvested them at 24 h, reporting that the Qingdai compound inhibited proliferation and promoted apoptosis in K562 cells. Then, the expression of bcr/abl and JWA was detected by semi-quantitative RT-PCR, and concentration-dependent decreases were found in bcr-abl and JWA expression of K562 cells. It was proved that the Qingdai compound can partially promote the apoptosis of K562 cells by inhibiting the expression of bcr/abl and JWA in K562 cells [8]; however, its specific mechanism needs further study. Therefore, it is necessary to develop a novel strategy to understand the biological processes of the interactions among drugs, genes, and proteins at a systems level in order to discover the molecular mechanisms related to the therapeutic efficacy of TCM.

In recent years, with the continuous innovation and development of systems biology, network pharmacology and molecular docking provide feasible research strategies for exploring the intrinsic principles of effective intervention of traditional Chinese medicine (TCM) components and building multi-target precise treatment modes for TCM [9,10]. It has been successfully applied to the molecular network level understanding of the pharmacological mechanism of TCM. For example, in the treatment of diabetes mellitus, Huangqi and Huanglian showed the synergistic mechanism [11], and through these research strategies we demonstrated the important pharmacological mechanism of Yin huang Qing fei capsule in treating chronic bronchitis [12].

We based the present study on network pharmacology strategies to decipher the pharmacological mechanisms of Qingdai acting on CML. We offer a systems strategy: (1) We collected the chemical components of Qingdai and downloaded structure and screening index data; (2) We predicted putative targets of Qingdai and analyzed putative targets by Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis; (3) We collected the known therapeutic targets of drugs in the treatment of CML; (4) We analyzed and investigated the network between putative targets of Qingdai and known therapeutic targets of CML, which provide a strategy for the further study of the pharmacological mechanism of Qingdai on CML; (5) We performed molecular docking between the molecular compounds of Qingdai and the major targets to validate our findings using a computer-aided drug design method. We expected to achieve our experimental goals with this series of experimental methods.

Material and Methods

The technical strategy of this research is shown in Figure 1.

Data preparation

Active compounds of Qingdai

Compositive compounds of Qingdai were obtained from TCMSP Database and Literature database. TCMSP (*http://ibts.hkbu.edu.hk/LSP/tcmsp.php*), updated in 2014-05-31) [13], which is based on the framework of systems pharmacology for herbal medicines, consists of all the 499 Chinese herbs registered in the Chinese pharmacopoeia with 29 384 ingredients and 12 important ADME-related properties are provided for drug screening and evaluation. Then, through literature mining to prevent omissions, we set the criteria of OB greater than 30%, DL greater than 0.18, and Caco-2 greater than -0.14. When they met these criteria, these components were used as candidate compounds for further analysis. We collected information on 7 compounds and obtained the name of the molecule and its chemical structure. We obtained the molecular Smiles

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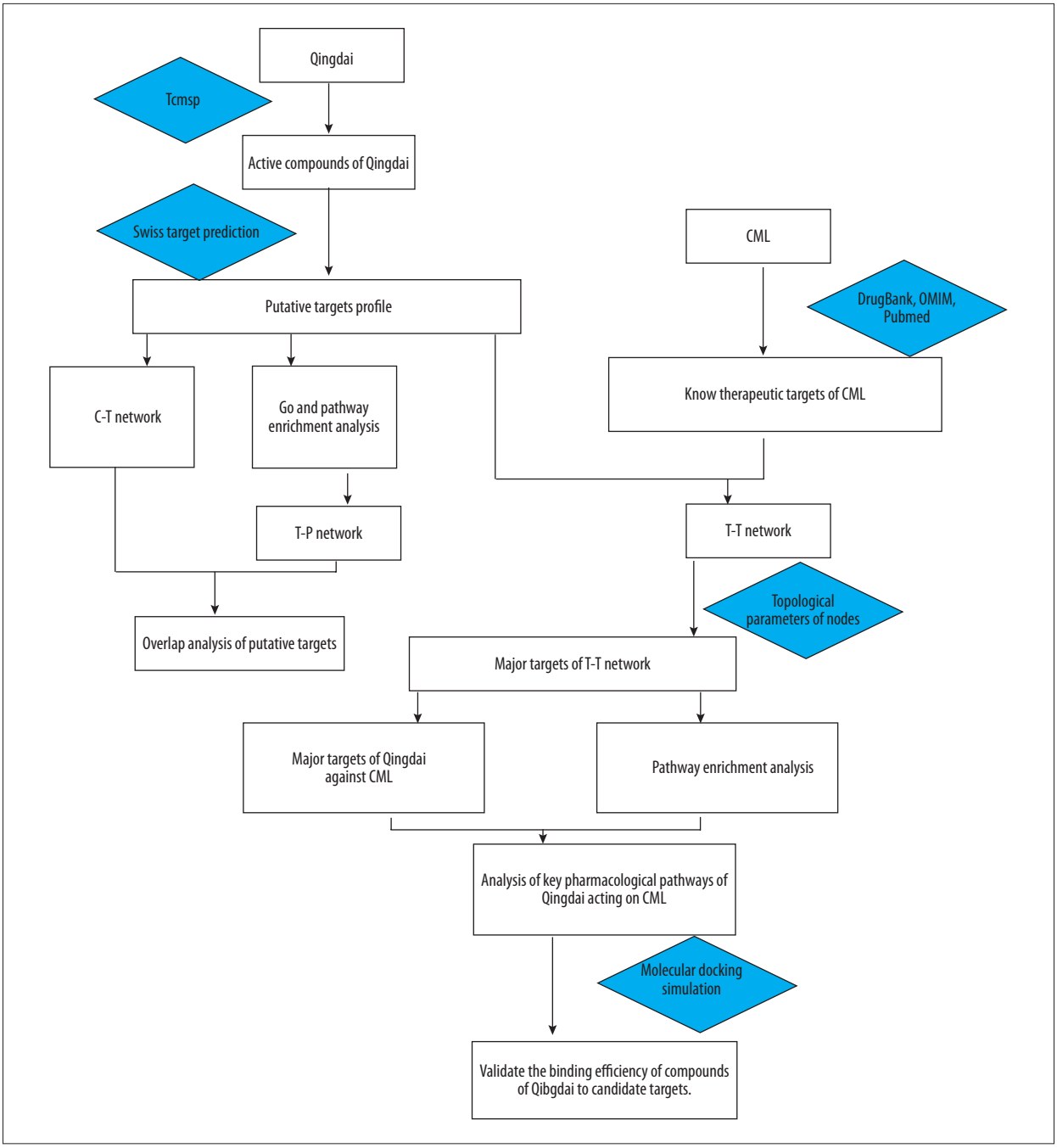

Figure 1. The technical strategy of this research based on network pharmacology for deciphering Key pharmacological pathways of Qingdai acting on CML.

format through the PubChem (*https://pubchem.ncbi.nlm.nih.gov/*) database.

Known therapeutic targets of drugs in the treatment of chronic myelocytic leukemia

The known therapeutic targets of drugs in the treatment of chronic myeloid leukemia were obtained in 3 ways: PubMed (*https://www.ncbi.nlm.nih.gov/pubmed*,2017-7-31), DrugBank20 (*http://www.drugbank.ca/*, version 5.0.10, released 2017-11-14), and the Online Mendelian Inheritance in Man (OMIM) database (http://www.omim.org/, released on 2017-12-20) [14]. In the PubMed database, "chronic myeloid leukemia" was retrieved, and the restriction was "gene" and "Homo sapiens." We verified the accuracy of the genes by consulting the literature related to these genes. In total, 252 known therapeutic targets

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of CML were chosen. In DrugBank, in order to improve accuracy, only the drugs that are approved by the Food and Drug Administration (FDA) and whose targets are human genes/proteins were selected, then we chose 265 targets for treating CML. In addition, when searching the OMIM database for "chronic myeloid leukemia" as a keyword, we collected 274 known therapeutic targets. After combining the data from these 3 databases and removing the duplicates, a total of 729 known targets for CML treatment were used for the next analysis. Supplementary Table 1 provides detailed information on these known therapeutic targets. We converted different types of ID proteins to UniProt IDs. To elucidate the signaling pathways involved in known therapeutic targets of CML, we used DAVID (Database Visualization and Integrated Discovery software, *http://david.abcc.ncifcrf.gov* version 6.7) and KEGG (Kyoto Encyclopedia of Genes and Genomes database, EGG, *http://www.genome.jp/kegg/*, updated on April 18, 2016) to perform enrichment pathways. The top 10 significant pathway terms were pathways in cancer, MAPK signaling pathway, natural killer cell-mediated cytotoxicity, Jak-STAT signaling pathway, cytokine-cytokine receptor interaction, chronic myeloid leukemia, prostate cancer, focal adhesion, ErbB signaling pathway, and neurotrophin signaling pathway.

Prediction of targets of Qingdai

Obtaining the target of Qingdai through experiments requires a great deal of manpower, material, and financial resources. To accurately predict the targets of bioactive molecules based on a combination of 2D and 3D similarity measures with known ligands, we used the web server Swiss Target Prediction (*http://www.swisstargetprediction.ch/*) to predict the putative targets of the active compounds of Qingdai. Predictions can be carried out in 5 different organisms, and mapping predictions by homology within and between different species is enabled for close paralogs and orthologs [15]. The "smiles" formats of 7 active compounds were imported into Swiss Target Prediction to predict their putative targets of action. It is noteworthy that the predicted putative target is limited to Homo sapiens, and to improve the reliability of predictions goal, only a high probability of target selected. A total of 112 therapeutic putative targets were obtained. All putative targets obtained were sent to Therapeutic Target Database (TTD) (*http://bidd.nus.edu.sg/group/cjttd/*, 2015-09-10), Comparative Toxicogenomics Database (CTD) (*http://ctdbase.org/*, 2017-12-05), and PharmGKB (*https://www.pharmgkb.org/*) to determine whether these putative targets have some connection to CML. To further understand the putative target of Qingdai, GO enrichment analysis and KEGG pathways analysis were performed.

Network construction

Three types of visual networks were built:

1) The compound-target network (C-T network) is an interaction network using the active compounds of Qingdai and its corresponding putative targets.
2) The target-pathway network (T-P network) is composed of the putative targets and corresponding pathways.
3) The target-target network (T-T network) was built using the relationship between the putative targets of Qingdai and known therapeutic targets of the CML.

Cytoscape 3.5.1 (*http://www.cytoscape.org/*) is an open software application for visualizing, integrating, modeling, and analyzing interactive networks. All the networks were built using it.

Analysis of the target-target network (Qingdai putative target-known therapeutic targets of the CML network).

Li et al. [16] suggested that "If the degree of a node is more than 2 times the median degree of all the nodes in a network, the node may function as a big hub." The topological features of the target-target network are analyzed by several important topological properties, such as "degree" [17], "betweenness" [17], "closeness" [17], and "coreness" (an iterative process in which nodes are removed from the network with minimal connection order) [18]. The larger a protein's degree/node betweenness/closeness centrality, the more important that protein is in the PPI network [19]. Subsequently, the targets were screened for topological importance. Then, the major hubs were screened. The DAVID webserver was used to perform KEGG pathway enrichment analysis of the main targets.

Molecular docking simulation

We used computer molecular docking simulation techniques to verify the credibility of the study. SystemsDOCK (*http://systemsdock.unit.oist.jp/*) was used for molecule docking [20]. SystemsDock is a web server for network pharmacology-based prediction and analysis that permits docking simulation and molecular pathway mapping for comprehensive characterization of ligand selectivity and interpretation of ligand action on a complex molecular network. All the compounds and 3D structures of Qingdai were directly downloaded from the PubChem database (*https://pubchem.ncbi.nlm.nih.gov/*, 2017-11-26), and we obtained the 3D structures of target genes from Uniprot (*http://www.uniprot.org/*, 2017-11) and PDM databases (*http://www.rcsb.org/pdb/home/home.do*). Docking scores were used to assess the binding affinities of compounds to the respective candidate target.

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Results

Active compounds in Qingdai

A single Chinese medicine contains a large number of compounds, so it is helpful to identify these active compounds by means of network pharmacological virtual screening. A total of 53 compounds in Qingdai were obtained. Then, 3 ADME (absorption, distribution, metabolism, and excretion)-related models, including OB, DL, and Caco-2, were used to screen most of the active compounds from Qingdai. Finally, we selected 7 compounds from Qingdai (Table 1), and after text mining, found that most of these compounds possess potent pharmacological activities, such as indirubin, the main active and characteristic compound in Qingdai. Research shows that indirubin and its derivatives can be used to treat chronic myelogenous leukemia by potently inhibiting the Signal Transducer and Activator of Transcription 5 (Stat5) protein in CML cells [21], and indirubin and its derivatives could have anti-angiogenic activity [22]. Studies on Qingdainone have shown anti-tumor and anti-inflammatory effects [23]. Quindoline can cause cell cycle arrest, resulting in inhibition of cell proliferation and causing cell apoptosis [24]. Bisindigotin was found to dose-dependently inhibit TCDD-induced ethoxyresorufin O-demethylase (EROD) activity to achieve an anti-tumor effect [25]. Isoindigo can mediate the cell proliferation pathway to promote apoptosis [26,27]. Beta-sitosterol could inhibit the growth of bacteria and was found to be anti-inflammatory [28]. Indirubin and Indigotin were determined to be the quality markers of Qingdai in the Chinese Pharmacopoeia (The State Pharmacopoeia Commission of China, 2015).

Putative targets of Qingdai

For Qingdai, through putative target prediction for the 7 components, a total of 112 targets were obtained. Cyclin-dependent kinases (CDKs) are involved in regulating both cell cycle and transcription. Indirubin inhibits CDK activity by K562 cell cycle arrest and promotes apoptosis [29,30]. With Quindoline, through prediction, MAPKs (mitogen- activated protein kinase) and CLKs were obtained. MAPKs play key roles in many cell proliferation-related signaling pathways [31]. Research by Ahmed K found in cancer cells that CLKs control the supply of full-length, functional mRNAs coding for a variety of proteins essential for cell growth and survival. Thus, inhibition of CLKs might become a novel anticancer strategy, leading to a selective depletion of cancer-related proteins after turnover [17]. β-sitosterol has antioxidant activity in a complex system [32]. Interestingly, 28 of the 112 putative target genes are common targets for one or more of these components, indicating that these components may be acting on some of the same biological processes or pathways, which reflects a synergistic effect between the individual components of TCM.

The C-T network was constructed to visualize and explain the complex relationship between the active compounds of Qingdai and its putative targets (Figure 2).

GO enrichment and KEGG pathway analysis of the putative targets

The GO and KEGG enrichment analysis were used to comment on the 112 putative targets of Qingdai. As shown in the results of the enrichment, a total of 433 GO enrichment results were obtained, including biological process (BP) (310 terms), molecular function (MF) (86 terms), and cellular component (CC) (38 terms). We set the level of statistical significance at $P<0.05$. Then, the top 10 significantly enriched terms were selected in the BP, MF, and CC categories listed in Figure 3. GO enrichment analysis showed that Qingdai can inhibit protein kinase phosphorylation and protein kinase to inhibit cell proliferation, block the cell signaling pathway to inhibit cell proliferation, and promote apoptosis. In addition, chemokines inhibit tumor growth and development by activating immunocompetent cytotoxic cells or inhibiting tumor-associated angiogenesis. In addition, Qingdai can be organized by cell division cycle of proliferation to inhibit cell proliferation or cell mitosis. In addition, it acts on GPCRs, which are closely related to biological behaviors such as the proliferation, invasion, and metastasis of tumors, involving the classical signal pathways such as ERK/MAPK [33]. In recent years, studies have shown that it can serve as a new target for anti-tumor drugs [34]. It is possible that the role of Qingdai on CML is through these molecular mechanisms.

The putative targets of active compounds were mapped onto the 26 KEGG pathways (Figure 4). The neuroactive ligand-receptor interaction pathway showed the highest number of target connections (count=13), and cytokine-cytokine receptor interaction with 12 targets, pathways in cancer with 11, and included the focal adhesion, cell cycle, chemokine signaling pathway, MAPK signaling pathway, and p53 signaling pathway, respectively. These pathways have well-established roles in the inhibition of tumor cell growth and differentiation and promote tumor cell apoptosis. In addition, there are numerous signaling pathways involved in immunity and inflammation, such as Toll-like receptor signaling pathways, T cell receptor signaling pathway, and Fc epsilon RI signaling pathway. These pathways play an important role in the infection caused by chronic myeloid leukemia. These pathways of the targets show that Qingdai has a therapeutic effect for a variety of malignant tumors, endocrine disease, and inflammatory diseases. Details are provided in Table 2.

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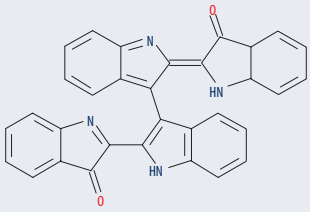
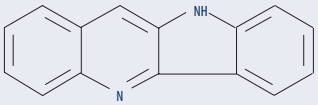
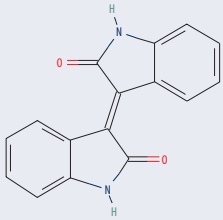
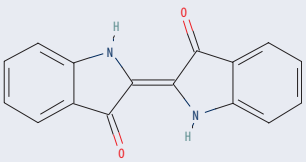
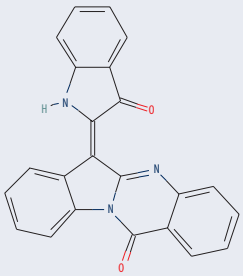
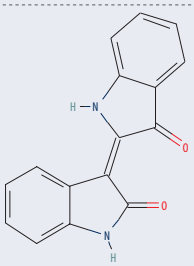
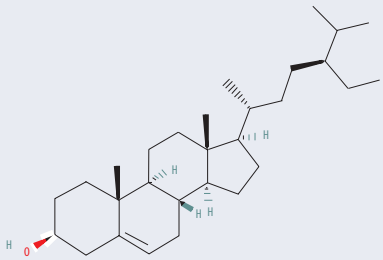
Table 1. Active compounds and ADME parameters of Qingdai.

No	Name	Structure	OB (%)	DL	Caco-2
MOL011100	Bisindigotin		41.66	0.39	0.90
MOL011332	Quindoline		54.57	0.22	1.52
MOL011335	Isoindigo		94.30	0.26	0.79
MOL001781	Indigotin		38.20	0.26	0.83
MOL001810	Qingdainone		45.28	0.89	1.19
MOL002309	Indirubin		48.59	0.26	1.26
MOL000358	Beta-sitosterol		36.91	0.75	1.32

OB – oral bioavailability; DL – druglikeness; Caco-2 – Caco-2 permeability.

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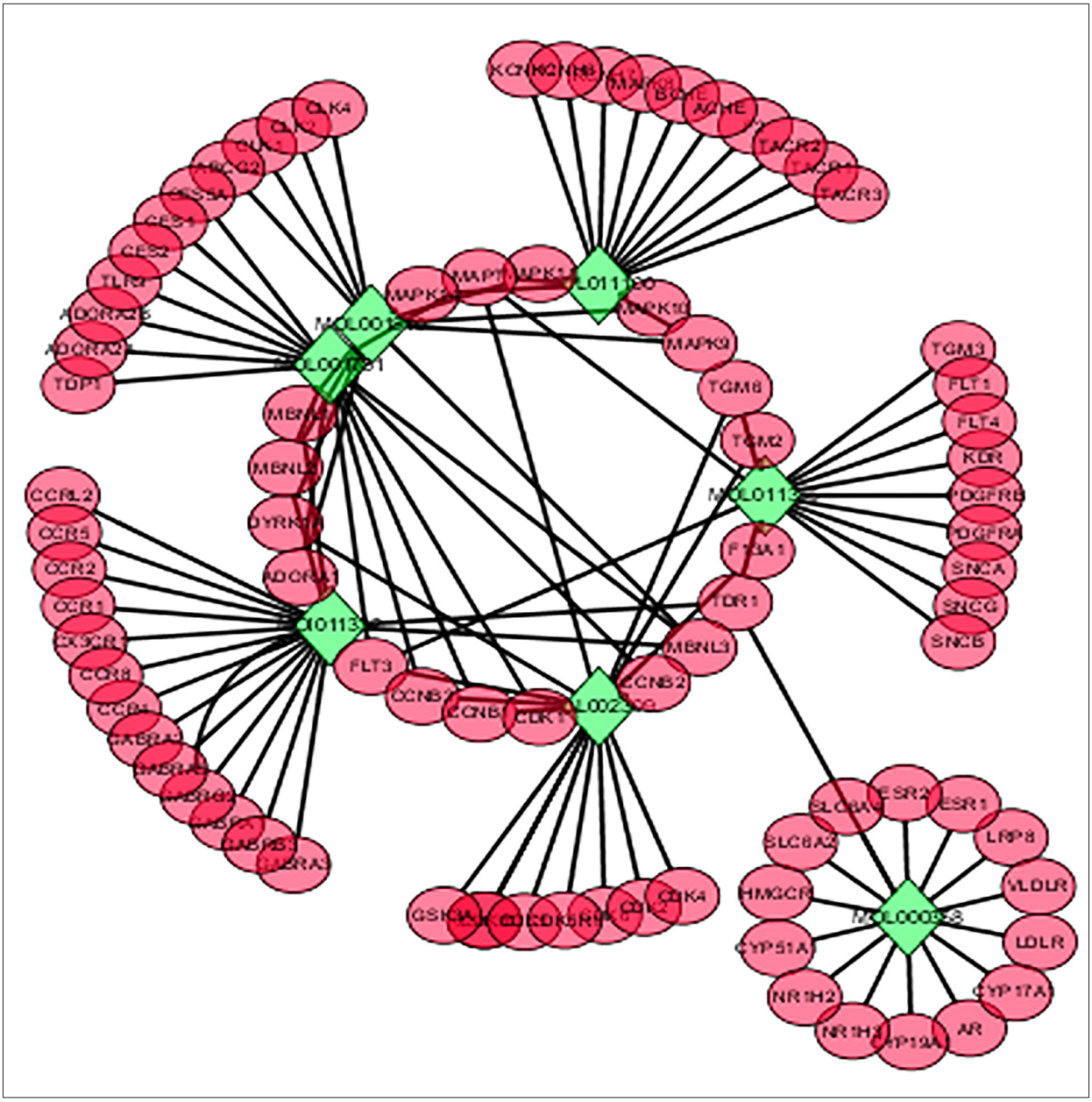

Figure 2. Compound-Target network (C-T network). Network of 7 active compounds of Qingdai and 112 putative targets.

Pharmacological mechanisms of Qingdai acting on chronic myeloid leukemia

The link between traditional Chinese medicine and disease is complex. To illustrate the basic relationship between them, the T-T network was performed for analysis. T-T network consisted of 571 nodes and 10 169 edges. The major hubs in the hub interaction network were determined by calculating 4 features: "degree," "node betweenness," "closeness", and "K value". There were 195 major hubs, including 32 Qingdai targets (Table 3) and 168 known therapeutic targets of chronic myeloid leukemia. Interestingly, there were 11 targets that were common to both that were screened. Then, a network of major hubs based on their direct interactions was constructed (Figure 5).

To further decipher the pharmacological mechanism by which Qingdai affects CML, pathway enrichment analysis was performed using the KEGG pathway database. We found that the major hubs were significantly related to various physiological processes, mainly concentrated in 5 annotation clusters, including epidermal growth factor receptor signaling pathways for cell growth, proliferation, differentiation and metabolism, malignant pathways, immune and inflammation-related pathways,

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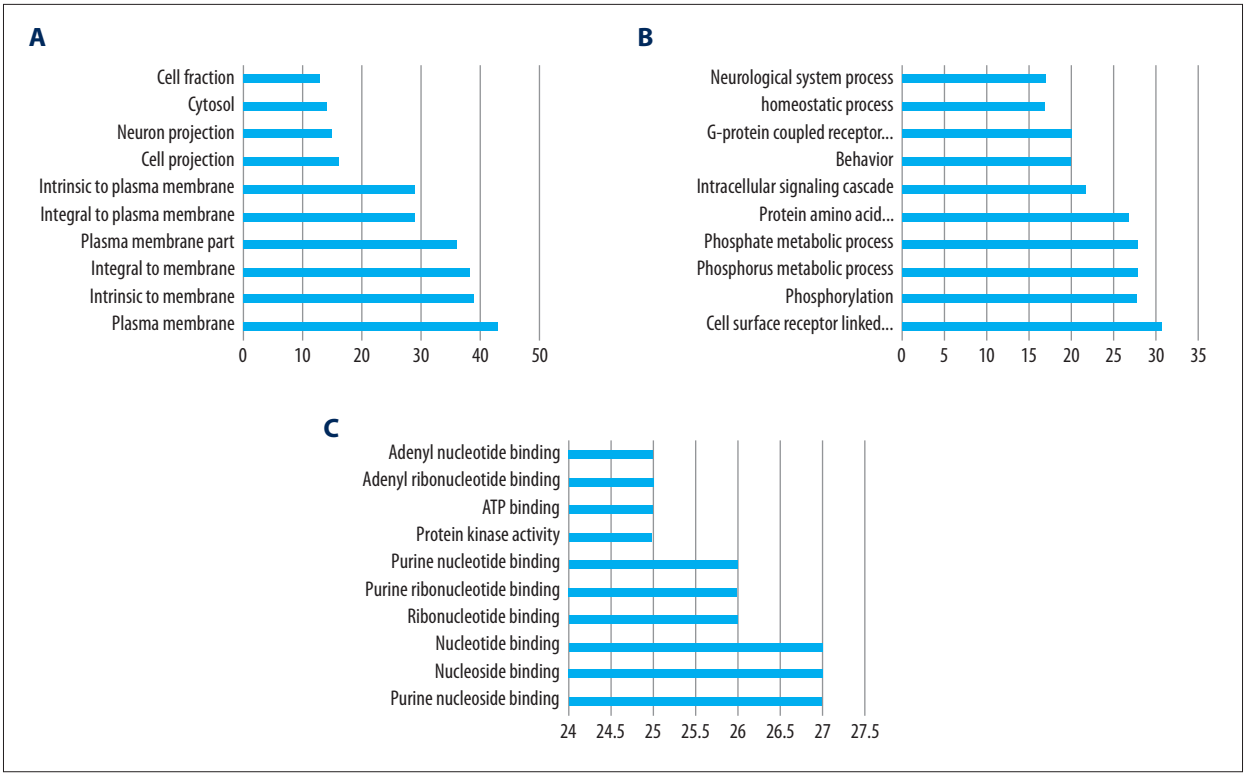

Figure 3. GO enrichment analysis of the putative targets of Qingdai. The top 10 significantly enriched terms in CC, BP, and MF categories. Cellular component (**A**), Biological process (**B**), Molecular function (**C**).

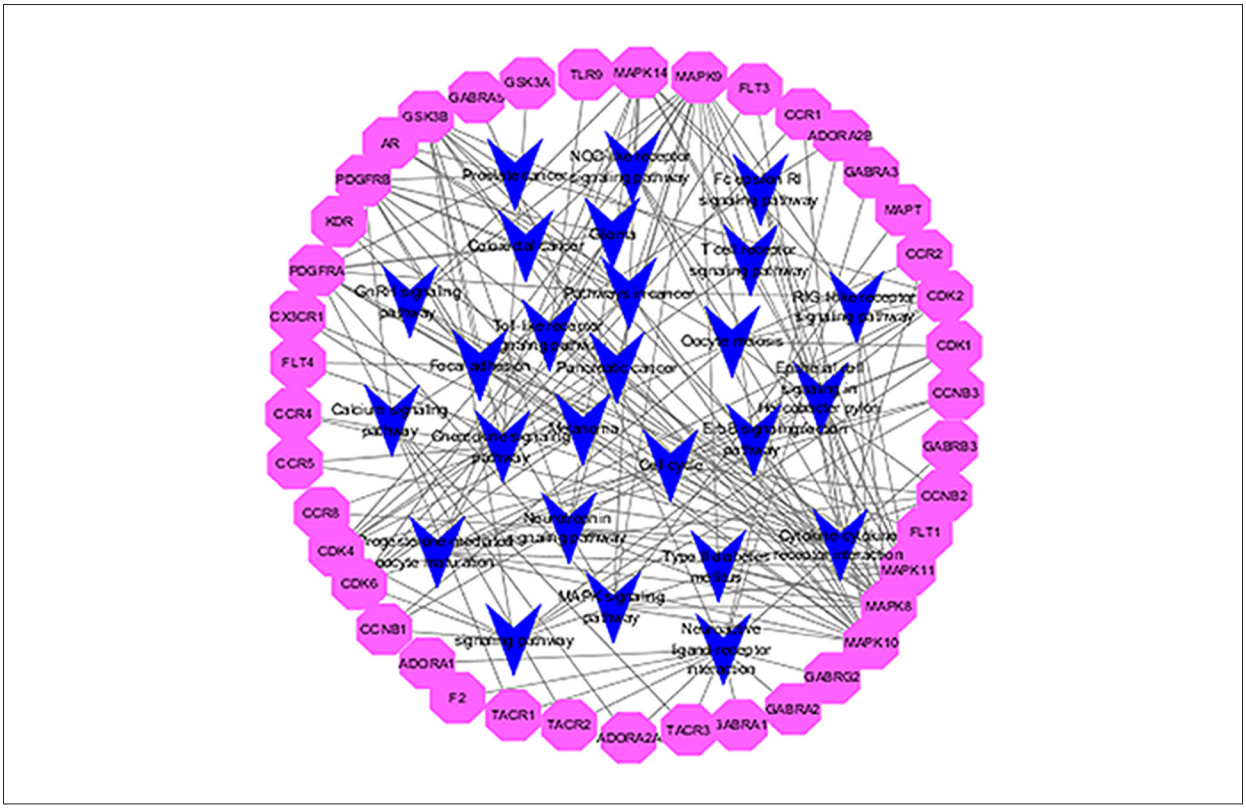

Figure 4. The network of putative targets of Qingdai and 26 KEGG pathways.

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Table 2. The 26 KEGG pathways associated with the putative targets of Qingdai.

Term	Count	P-value
hsa04914: Progesterone-mediated oocyte maturation	10	2.93E-07
hsa04080: Neuroactive ligand-receptor interaction	13	1.57E-05
hsa04115: p53 signaling pathway	7	8.78E-05
hsa04060: Cytokine-cytokine receptor interaction	12	1.03E-04
hsa04110: Cell cycle	8	3.91E-04
hsa04510: Focal adhesion	9	0.001437085
hsa05210: Colorectal cancer	6	0.002152457
hsa05200: Pathways in cancer	11	0.002692876
hsa04062: Chemokine signaling pathway	8	0.004097191
hsa04621: NOD-like receptor signaling pathway	5	0.004583166
hsa04620: Toll-like receptor signaling pathway	6	0.004793465
hsa05120: Epithelial cell signaling in *Helicobacter pylori* infection	5	0.006372769
hsa04622: RIG-I-like receptor signaling pathway	5	0.00742055
hsa05212: Pancreatic cancer	5	0.007793627
hsa04664: Fc epsilon RI signaling pathway	5	0.010293415
hsa04722: Neurotrophin signaling pathway	6	0.011255823
hsa04020: Calcium signaling pathway	7	0.012206329
hsa05215: Prostate cancer	5	0.016120746
hsa04912: GnRH signaling pathway	5	0.022181176
hsa04010: MAPK signaling pathway	8	0.026015669
hsa04660: T cell receptor signaling pathway	5	0.030365615
hsa05214: Glioma	4	0.03155056
hsa04114: Oocyte meiosis	5	0.032191066
hsa05218: Melanoma	4	0.042714992
hsa04012: ErbB signaling pathway	4	0.040141341
hsa04930: Type II diabetes mellitus	3	0.034045303

and angiogenesis-related pathways. Chronic myeloid leukemia is a malignant proliferative disease of bone marrow hematopoietic cells and is closely related with ErbB receptor overexpression [35]. ErbB receptor signaling regulates cell proliferation, migration, differentiation, apoptosis, and cell migration through Akt, MAPK, and many other pathways. In many forms of malignancy in organs such as the breasts, ovaries, brain, and prostate gland [36], members of the ErbB family, as well as some of their ligands, are often overexpressed, amplified, or mutated, making them an important therapeutic target [37]. Immune and inflammatory signaling pathways include the Toll-like receptor signaling pathway, T cell receptor signaling pathway, B cell receptor signaling pathway, and Fc epsilon RI signaling pathway. TLR activation has been described to play a role in other leukemias, such as chronic lymphocytic leukemia [38]. T cell receptor (TCR) activation can promote many signal transduction cascades and ultimately determine cell fate by regulating cytokine production, cell survival, proliferation, and differentiation [39]. Regulatory T (Treg) cells can weaken anti-tumor immune responses, which could serve as a promising immuno-therapeutic approach for tumors [40]. The Fc epsilon RI receptor induces multiple signaling pathways that control the secretion of allergic mediators and induction of cytokine gene transcription, resulting in secretion of various molecules: IL-4, IL-5, IL-6, IL-10, IL-13, INF-gamma (interferon-gamma), and TNF-alpha (tumor necrosis factor alpha) [41]. We provide detailed information of the 20 most meaningful enrichment pathways in Table 4.

Drug targets reported to be involved in CML pathogenesis for the treatment of CML are involved in cell cycle, growth inhibition, MAPK, ErBb, transforming growth factor beta, and p53 signaling pathways. Interestingly, the 32 Qingdai putative targets included in the major hubs of the T-T network were also

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Table 3. The 32 major targets information of Qingdai.

ID	Target	Uniprot ID	Gene name	PDB ID
MT-1	Mitogen-activated protein kinase 8	P45983	MAPK8	IUKH
MT-2	Estrogen receptor	P03372	ESR1	1A52
MT-3	Mitogen-activated protein kinase 14	Q16539	MAPK14	1A9U
MT-4	Cyclin-dependent kinase 2	P24941	CDK2	1AQ1
MT-5	Vascular endothelial growth factor receptor 2	P35968	KDR	1VR2
MT-6	Cyclin-dependent kinase 4	P11802	CDK4	2W96
MT-7	Androgen receptor	P10275	AR	1E3G
MT-8	Prothrombin	P00734	F2	1A2C
MT-9	Cyclin-dependent kinase 1	P06493	CDK1	4Y72
MT-10	Glycogen synthase kinase-3 beta	P49841	GSK3B	1GNG
MT-11	Platelet-derived growth factor receptor beta	P09619	PDGFRB	1GQ5
MT-12	Mitogen-activated protein kinase 9	P45984	MAPK9	3E7O
MT-13	G2/mitotic-specific cyclin-B1	P14635	CCNB1	2B9R
MT-14	Mitogen-activated protein kinase 11	Q15759	MAPK11	3GC8
MT-15	Receptor-type tyrosine-protein kinase FLT3	P36888	FLT3	1RJB
MT-16	Cyclin-dependent kinase 6	Q00534	CDK6	1BI7
MT-17	Vascular endothelial growth factor receptor 1	P17948	FLT1	1FLT
MT-18	Toll-like receptor 9	Q9NR96	TLR9	3WPB
MT-19	Cyclin-dependent-like kinase 5	Q00535	CDK5	1H4L
MT-20	C-C chemokine receptor type 5	P51681	CCR5	4MBS
MT-21	Alpha-synuclein	P37840	SNCA	2X6M
MT-22	Low-density lipoprotein receptor	P01130	LDLR	1AJJ
MT-23	Estrogen receptor beta	Q92731	ESR2	1L2J
MT-24	Glycogen synthase kinase-3 alpha	P49840	GSK3A	2DFM
MT-25	Aromatase	P11511	CYP19A1	3EQM
MT-26	Platelet-derived growth factor receptor alpha	P16234	PDGFRA	5GRN
MT-27	Mitogen-activated protein kinase 10	P53779	MAPK10	1JNK
MT-28	C-C chemokine receptor type 2	P41597	CCR2	5T1A
MT-29	Cyclin-dependent kinase 3	Q00526	CDK3	ILFN
MT-30	Microtubule-associated protein tau	P10636	MAPT	2ON9
MT-31	ATP-binding cassette sub-family G member 2	Q9UNQ0	ABCG2	5NJ3
MT-32	Vascular endothelial growth factor receptor 3	P35916	FLT4	4BSJ

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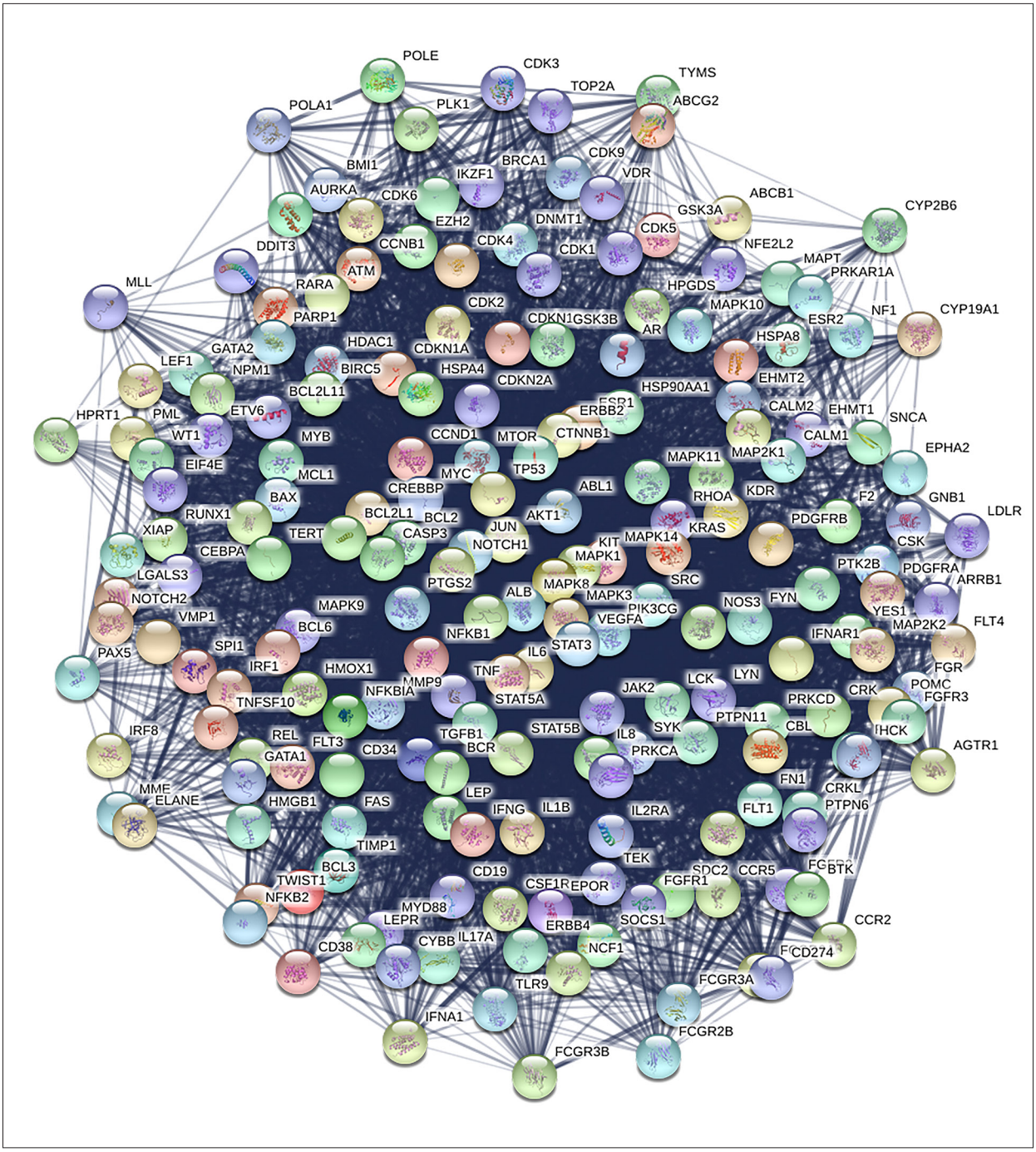

Figure 5. The network of 195 major hubs based on their direct interactions, consisting of 195 nodes and 5943 edges. Nodes represent proteins. Colored nodes are query proteins and first shell of interactors. White nodes are second shell of interactors. Empty nodes are proteins of unknown 3D structure. Filled nodes have some 3D structure known or predicted. Edges represent protein-protein associations and line thickness indicates the strength of data support.

included in these pathways. In addition, 32 putative targets were involved in immune and inflammation-related pathways, such as Toll-like receptor, NOD-like receptor, RIG-I-like receptor, and Fc epsilon RI T cell receptor signaling pathway.

To further explore the molecular mechanism of action of Qingdai on CML, we reviewed the literature on the role of Qingdai putative targets in these pathways. Qingdainone, bisindigotin, isoindigo, and indirubin all have target enrichment in the MAPK signaling pathway (MAPK14, MAPT, PDGFRA, PDGFRB,

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Table 4. The Top 20 enrichment pathways of 195 major hubs.

Term	Count	Value
hsa05200: Pathways in cancer	70	2.22E-41
hsa04010: MAPK signaling pathway	34	5.88E-12
hsa05220: Chronic myeloid leukemia	29	9.85E-24
hsa04062: Chemokine signaling pathway	28	1.71E-11
hsa04510: Focal adhesion	28	9.51E-11
hsa05215: Prostate cancer	27	5.69E-19
hsa04722: Neurotrophin signaling pathway	27	4.59E-15
hsa04012: ErbB signaling pathway	26	4.46E-18
hsa04060: Cytokine-cytokine receptor interaction	26	5.61E-07
hsa05210: Colorectal cancer	23	4.84E-15
hsa05221: Acute myeloid leukemia	22	1.16E-17
hsa04620: Toll-like receptor signaling pathway	22	2.94E-12
hsa04660: T cell receptor signaling pathway	22	1.16E-11
hsa04650: Natural killer cell mediated cytotoxicity	22	7.02E-10
hsa04630: Jak-STAT signaling pathway	22	1.23E-08
hsa05212: Pancreatic cancer	20	3.49E-13
hsa04664: Fc epsilon RI signaling pathway	19	1.82E-11
hsa04910: Insulin signaling pathway	19	1.88E-07
hsa05214: Glioma	18	4.43E-12
hsa04110: Cell cycle	18	3.16E-07

MAPK9, MAPK11, MAPK8, and MAPK10). CD Kang et al. showed that the inhibition of ERK/MAPK induced apoptosis in K562 cells [42]. PDGFRA/B are oncogenes involving tyrosine kinases [43]. Aberrant activity of PTK (protein tyrosine kinases) has been implicated in the stimulation of cancer growth and progression, the induction of drug resistance, tumor neovascularization, tissue invasion, extravasation, and the formation of metastases [44]. We speculate that isoindigo in Qingdai inhibits CML by acting on PDGFRA/B. In the ErbB pathway, GSK3B plays a pivotal role in preserving quiescent HSCs, which has now opened new therapeutic avenues for understanding leukemic stem cell function [45]. Through the cytokine-cytokine receptor interaction, cytokines act on the immune system and hematopoietic system and play an important regulatory role in cell–cell interactions, cell proliferation, differentiation, and effector functions [46]. The p53 protein network regulates important mechanisms in DNA damage repair, cell cycle regulation/checkpoints, and cell senescence and apoptosis, as demonstrated by its ability to positively regulate the expression of various pro-apoptotic genes [47]. In addition, research shows that p53 can stably induce CML cell apoptosis [48]. Cyclin-dependent kinases (CDKS) are a family of serine/threonine kinases that have been firmly established as key regulators of transcription processes underlying coordinated cell cycle entry and sequential progression in nearly all types of proliferative cells [49]. Infection with CML has important secondary symptoms. Enrichment pathways of Qingdai putative targets involve immune and inflammatory pathways, which activate the patient's own immune system and enhance the defence against sources of external infection, such as phagocytosis of immune cells, which plays an essential role in host defence mechanisms by enveloping and destroying infectious pathogens [41].

In addition, some of the putative targets have a special role in CML. The FMS-like tyrosine kinase 3 (FLT3) gene encodes a class III receptor tyrosine kinase (RTK) that plays important roles in the proliferation, differentiation, and survival of hematopoietic stem and progenitor cells (HSPCs), and FLT3 is frequently mutated and overexpressed in hematologic malignancies [50].

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The AGM130 compound is derived from indirubin, which is known as a CDK inhibitor. Research shows that the AGM130 compound efficiently decreased the viability of CML-derived K562 cells, which suggests that AGM130 is a strong candidate for treating Imatinib-resistant CML [51]. In addition, patients with ABCG2 diplotypes might be at higher risk for the rapid and severe development of CML and have a weaker response to treatments with imatinib [52]. We hypothesize that it binds to ABCG2 to enhance the efficacy and reduce the risk of imatinib resistance.

On this basis, the major putative targets of Qingdai that are significantly associated with these biological processes and pathways might play a role in the treatment of CML.

Molecular docking validation

Molecular docking is a rapid method to predict the binding force between traditional Chinese medicine components and the target. It is based on the docking of the ligand and the acceptor's spatial structure. SystemsDock applies AutoDock VINA [53] to perform docking simulation based on the characterized binding interaction and molecular properties [19]. DocK-IN utilizes a machine learning algorithm (Random Forest) together with a series of characterized binding interactions and test compound molecular properties, usually ranging from 0 to 10 (from weak to strong binding) allowing a straightforward indication of binding strength [20]. The 7 compounds of Qingdai and the corresponding candidate major targets were further validated by a molecular docking simulation. As a result, 23 pairs of components of Qingdai and candidate targets had binding efficiencies. Detailed information about the results of molecular docking are described in Supplementary Table 2. These findings require further experimental verification.

Discussion

In the application of traditional Chinese medicine treatment of CML, Qingdai is given high priority for selection, and has been frequently used in TCM prescriptions. *In vitro* experiments clearly demonstrated that Qingdai has the ability to inhibit K562 cell proliferation and promote its apoptosis. We used modern network pharmacology and molecular docking technology to explain the effective substance basis and multi-targeting effect of Qingdai treatment of CML. The study of traditional Chinese medicine theory and value is based on the scientific methodology of systematic medicine and has the significance of integrating innovation. In our research, we screened 7 Qingdai active compounds and, from a total of 112 predicted targets of active compounds, obtained 32 major targets of Qingdai for treatment of chronic myeloid leukemia, and enriched 15 signaling pathways related to the treatment of CML. Then, we verified the results of our study by molecular docking. The present study shows the following:

1) By predicting the targets of 7 compounds in Qingdai, we constructed a C-T network and performed GO analysis and KEGG analysis of the putative targets to provide clues to the pharmacology research of Qingdai.
2) We constructed the Qingdai putative target-known therapeutic targets of the CML network, suggesting that Qingdai may affect the disease-related pathways of chronic myeloid leukemia by regulating its candidate targets, such as the cytokine-cytokine receptor interaction, cell cycle, p53 signaling pathway, MAPK signaling pathway, and immune system-related pathways.
3) According to the molecular docking simulation, 23 pairs of components of Qingdai and corresponding putative targets had strong binding efficiencies.

Concusions

Network pharmacology for the study of complex mechanisms of Chinese medicine intervention disease provides new ideas and new methods. This research explored the molecular mechanism of the effects of Qingdai on CML based on these ideas. Our study was based on bioinformatics analysis and computer simulation analysis. Further clinical application assessments and experimental validations for these predicted results are required.

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Supplementary Tables

Supplementary Table 1. known therapeutic targets of CML.

ESR1	MLL	IMD21	QACR	SLC2	LY75	SFRP1	PTK2B
TP53	HRX	MME	ACRB	SLCO1B1	CBY1	LEF1	ELANE
BCR	HTRX1	CD10	CNGA1	SLC22A8	ARHGAP26	DDIT3	
	WDSTS	CALLA	KCNMA1	POLB	ST3GAL1	GSTA1	PTCH1
ABCB1	CBL2	NEP	CLCN2	CDA	MIR199B	MECOM	CD247
MTHFR	NSLL	CMT2T	IL17A	NT5E	ST8SIA4	SRSF1	STAT5B
TNF	CLLS5	SCA43	ADRB1	DCTD	ATG4B	CDKN1C	AICDA
JAK2	FLI1	MLF1	TUBB1	ABCC10	MIRLET7I	MIR17	IDH2
IL6	BDPLT21	LPP	CYP2E1	SLC29A1	PTBP2	CRK	AXL
TGFB1	ETV6	CHIC2	VDR	1KBKG	SPRED2	GATA2	CDK9
AKT1	TEL	BTL	MPL	PRKCA	OSBP2	NOTCH2	PMP22
GSTM1	THC5	PBT	CHRM1	IMPDH1	DDX43	PRTN3	ADIPOR1
CTNNB1	KRAS2	RAP1GDS1	CNRM2	IMPDH2	P2RX5	IL32	IKZF1
KRAS	RASK2	TCS1	BCHE	PB1	MKNK2	MIR223	TET2
GSTT1	NS	BST2	UREC	NT5C2	UBASH3B	IL24	MTHFD1L
NFKB1	CFC2	DKCA2	MMP12	ENPP1	MIR30E	ARRB1	NFKB2
BRCA1	RALD	DKCB4	NS5B	PRKCD	ATP5F1	PAX5	ERCC5
MMP9	CMTS	PFBMFT1	ADRB2	MS4A1	KIR2DL5B	WSB1	DAPK1
STAT3	PTPN11	CMM9	PTAFR	HCK	GAS2	CIP2A	POU2F1
ABL1	PTP2C	LIFR	HRH1	CDK2	FAM27E5	KIF11	SPI1
PTGS2	SHP2	SWS	NS5A	MAP2K1	ETNK1	MIR31	SLC22A1
CDKN2A	NS1	STWS	ADRA2A	MAP2K2	MIR1301	BIN1	SEP9
IL1B	JMML	SJS2	ADRA2B	MAP3K2	ST8SIA6	SET	
MYC	METCDS	ACSL6	CHD1	1FNAR2	LOC107126288	JUP	
GSTP1	CLLS2	FACL6	DRD2	POLA1	LOC107126281	REL	
CXCL8	D13S25	ACS2	DRD1	SLC28A3	MIR564	HOXA9	
LEP	DBM	IRF1	HTR2A	PARP1	MIR2278	CRKL	
TERT	FLVCR2	MAR	OPRM1	PARP2	MIR4701	CSF3R	
BCL2	C14orf58	GRAF	PDE4B	PARP3	ABL	SLCO1B3	
IFNG	CCT	PDGFR	PANX1	CD22	GF1R	MTHFD1	
MTOR	PVHH	IBGC4	CYP3N4	POLE	SPC	IFNAR1	
XRCC1	EPV	IMF1	CASR	POLE2	EPHA2	EHMT2	
FAS	SERPINA1	PENTT	KCND	POLE3	ICK	CDC6	
CCND1	PI	KOGS	HTR7	POLE4	YES1	ADIPOR2	
BIRC5	AAT	NSD1	ORM2	PNP	KIT	PER3	
GSK3B	TCL1B	ARA267	SLC6A2	C3	FYN	KIR2DS2	
MAPK14	TML1	STO	SMPD1	C4A	BTK	EPHB4	
ATM	TCL1A	SOTOS1	HTR1A	C4B	NR4A3	MEF2C	
MIR21	TCL1	CLLS4	NOMO1	C5	CSK	ASXL1	
HMGB1	MYL	DEK	MAP2	AOX1	EPHA5	HES1	

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CYP1A1	CREBBP	D6S231E	PHD	DNMT1	FGR	KIR2DL2
NOTCH1	CBP	CTEPH1	CA2	SLC01A2	FRK	PRAME
KDR	RSTS1	HLA-B	NR1I2	HSD11B1	HSPA8	SMO
NFE2L2	MYH11	SPDA1	POMC	ALB	ZAK	ORM1
HLA-G	AAT4	HLA-DPB1	CALM1	RARA	PPAT	MSR1
CASP3	FAA4	TREM2	YARS	RARB	CYP3A4	NUMB
ABCG2	FUS	MYB	TAT	1KBKB	CYP1A2	CAMK2G
EZH2	TLS	ALL2	SLC16A2	TXNRD1	CYP1B1	CCDC170
CDKN1B	ALS6	MLSM7	TFPI	MAPK3	FM03	GPX3
JUN	ETM4	DEL7q	GNRHR	MAPK1	RET	MIR451A
ERCC2	CBFB	C7DELq	KCNQ2	CDKN1A	NTPK1	CDK7
TNFSF10	PEBP2B	NCF1	KCNQ3	HDAC1	CSF1R	MIR101-1
RHOA	DIA4	PRSS2	HTR3A	PML	DDR1	IRF8
WT1	NMOR1	TRY2	GRIA1	ADA	CYP3A7	KIR2DS4
PDCD1	CYBA	SCLL	GNB1	CD38	CYP2C9	ID4
LGALS3	NF1	NSD3	MRD42	CD19	CYP2D6	CD33
CYP3A5	VRNF	WHSC1L1	CTRC	RXRA	CYP2C19	KIR2DS1
CD274	WSS	SLC20A2	CLCR	RXRG	PTGS1	HMMR
KCNH2	NFNS	MLVAR	IMD22	1GFBP3	SLC22A2	SOCS2
LCN2	ERBB2	GLVR2	TPOR	PSG5	ABCA3	BLK
AURKA	NGL	IBGC1	MPLV	CSF2RA	CYP2C8	RMND1
RUNX1	NEU	NBN	THCYT2	1L3RA	UGT1A1	MIR224
LEPR	HER2	NBS1	TAL1	SDC2	GSTA2	RANGAP1
HSP90AA1	MSF	THCYT3	TCL5	PRG2	MGST2	FUT1
NPM1	MSF1	LALL	SCL	EPOR	FLT3	PCM1
AKAP12	NAPB	BSAP	BCL10	GPRC5A	RPI2	MIR130A
BCL2L1	SH3GL1	ALL3	IMD37	NROB1	RPL3	MIR7-1
MCL1	EEN	TAL2	GFI1	ALDH1A2	TEK	AHI1
IL2RA	LYL1	CLLS3	ZNF163	RARRES1	FGFR1	CDKN2C
PLK1	CEBPA	CHDSKM	SCN2	LCN1	FGFR2	ZBTB2
PTPN22	CEBP	NUP214	RBM15	OBP2A	FGFR3	PLCD1
XIAP	BCL3	D9S46E	SPEN	RBP4	FGFR4	MTSS1
BCL2L11	BAX	CAN	OTT	PDK4	LCK	MSI2
DPP4	TAM	CAIN	IGFR2	CYP26A1	SRC	FERMT3
SYK	MST	AF10	CD32	HPGDS	ABCB11	PIWIL1
PDGFRA	CBFA2	ALL1	PBX1	ATP1A1	FCGR3B	RAPGEF1
MIR155	AML1	MBL2	CAKUHED	DGUOK	C1R	MKNK1
TWIST1	CML	MBL	ABL2	1TGAL	C1QA	EHMT1
CBL	PHL	MBP1	ABLL	TOP2A	C1QB	USF2
STAT5A	ALL	MBL2D	ARG	PDLA1	C1QC	MIR10A
BMI1	HMOX1	MBPD	NCF2	CBR1	FCGR3A	IL1RAP
HSPA4	HMOX1D	LMO1	FLVCR1	AKR1A1	C1S	MIR320A
TGM2	NCF4	RBTN1	AXPC1	AKR1	FCGR1A	LTB4R2
NFKBIA	P40PHOX	RHOM1	PCARP	NQO1	FCGR2A	SETBP1

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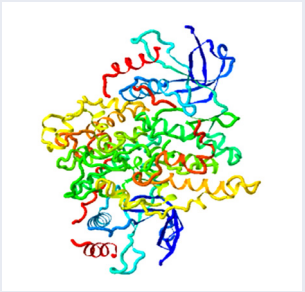
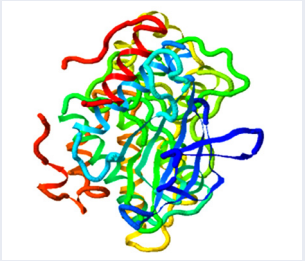
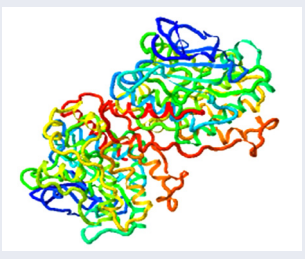
CD34	CGD3	LMO2	COPD	NOS3	FCGR2B	ULBP2
CALR	MKL1	RBTNL1	TCL4	NDUFS3	FCGR2C	BTG1
MIR34A	AMKL	RHOM2	ERBB4	NDUFS7	RRM2	KDM5A
XRCC3	MAL	TTG2	HER4	POR	DCK	MLLT3
PDGFRB	XK	CLLS1	ALS19	ABCC3	PRKAR2A	PHF6
SOCS1	MCLDS	SMAR	SGOL1	HPRT1	PRKAR1A	CD7
ABCC1	CYBB	NUMA1	SGO	TUBB	PDE3A	PTPRG
IFNA1	CGD	PICALM	SGO1	TUBA4A	1FNAR1	FCER1G
PIK3CG	AMCBX2	CALM	CAID	DHFR	FNTB	HULC
CYP2B6	IMD34	CLTH	THRB	FPGS	PDGFD	MIR196B
EIF4E	GATA1	LAP	ERBA2	TYMS	FLT1	MR1
XPC	GF1	ATA	THR1	ATIC	FLT4	RIN1
AURKB	ERYF1	AT1	PRTH	GGH	UGT1A3	ST3GAL4
PTPN6	NFE1	ZBTB16	MYD88	FOLR1	UGT1A4	FIP1L1
DNMT3A	XLTDA	ZNF145	MYD88D	IFNAR2	UGT1A9	MIR148B
BCL6	XLTT	PLZF	DCML	NS3	UGT2B7	MIR326
ALOX5	XLANP	KMT2A	MONOMAC	4A	UGT2B15	MIR486-1

Supplementary Table 2. Molecular docking between the 7 compounds of Qingdai and the corresponding candidate major targets.

Compounds and targets	Protein-ligand interactions of the docking pose	Score
Qingdainone, MAPK9		4.306
Qingdainone, MAPK10		4.684
Qingdainone, MAPK11		4.435

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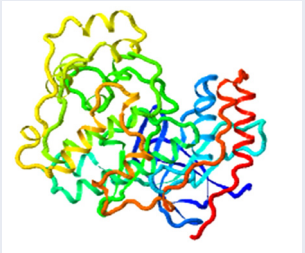
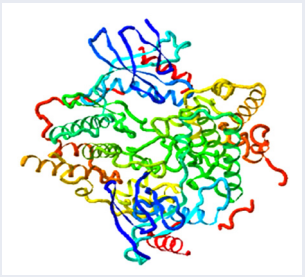
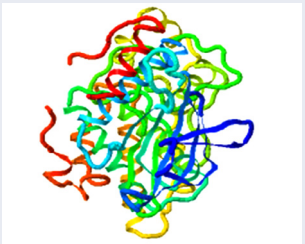
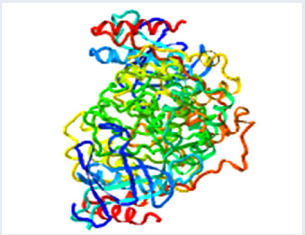
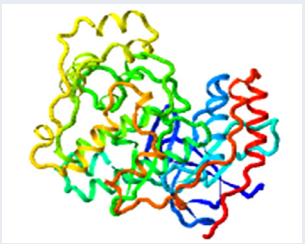
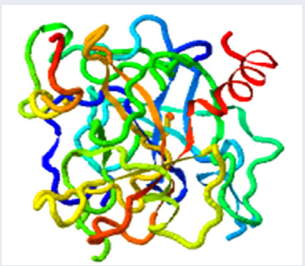
Compounds and targets	Protein-ligand interactions of the docking pose	Score
Qingdainone, MAPK11		4.844
Bisindigotin, MAPK9		4.053
Bisindigotin, MAPK10		3.562
Bisindigotin, MAPK11		3.787
Bisindigotin, MAPK14		4.039
Bisindigotin, F2		4.598

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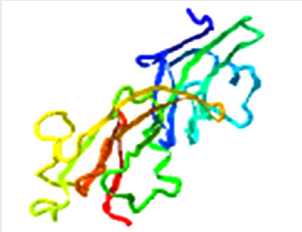
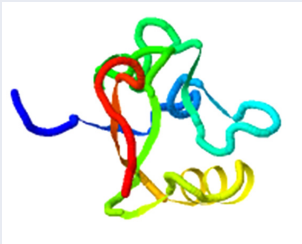
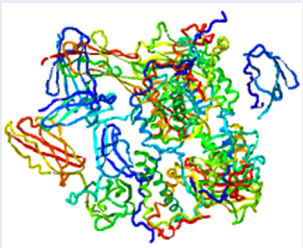
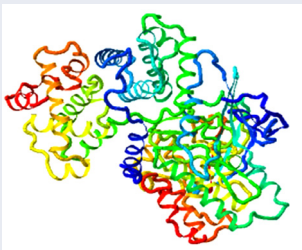
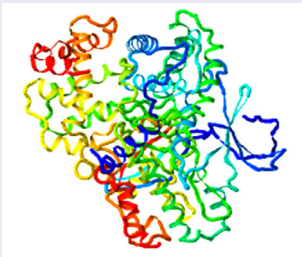
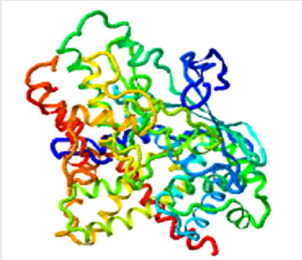
Compounds and targets	Protein-ligand interactions of the docking pose	Score
Isoindigo, FLT4		7.117
Isoindigo, PDGFRB		5.516
Isoindigo, FLT3		7.780
Indigotin, CDK1		6.860
Indirubin, CDK1		2.633
Indirubin, CDK4		2.051

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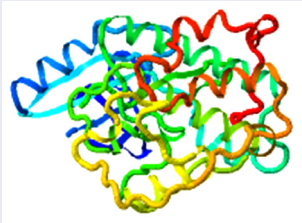
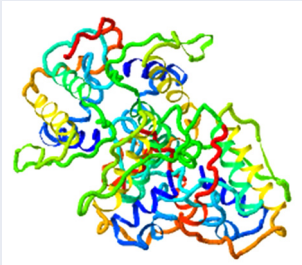
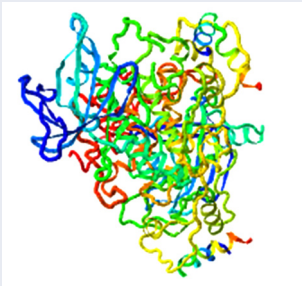
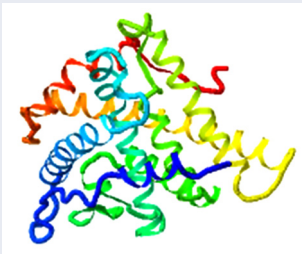
Compounds and targets	Protein-ligand interactions of the docking pose	Score
Indirubin, CDK2		2.583
Indirubin, FLT3		3.217
Indirubin, GSK3B		1.963
Beta-sitosterol,AR		8.365
Beta-sitosterol, CYP19A1		8.335

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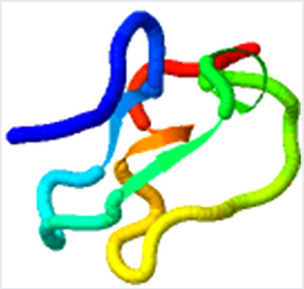
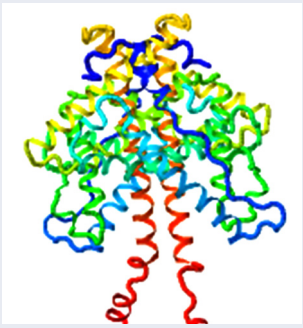
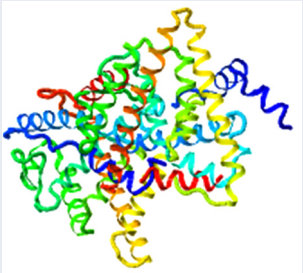
Compounds and targets	Protein-ligand interactions of the docking pose	Score
Beta-sitosterol, LDLR		4.981
Beta-sitosterol, ESR1		8.372
Beta-sitosterol, ESR2		8.321

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