Deciphering the mechanism of Indirubin and its derivatives in the inhibition of Imatinib resistance using a “drug target prediction-gene microarray analysis-protein network construction” strategy

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Abstract

Background: The introduction of imatinib revolutionized the treatment of chronic myeloid leukaemia (CML), substantially extending patient survival. However, imatinib resistance is currently a clinical problem for CML. It is very important to find a strategy to inhibit imatinib resistance.

Methods: (1) We identified indirubin and its derivatives and predicted its putative targets; (2) We downloaded data of the gene chip GSE2810 from the Gene Expression Omnibus (GEO) database and performed GEO2R analysis to obtain differentially expressed genes (DEGs); and (3) we constructed a P-P network of putative targets and DEGs to explore the mechanisms of action and to verify the results of molecular docking.

Result: We identified a total of 42 small-molecule compounds, of which 15 affected 11 putative targets, indicating the potential to inhibit imatinib resistance; the results of molecular docking verified these results. Six biomarkers of imatinib resistance were characterized by analyzing DEGs.

Conclusion: The 15 small molecule compounds inhibited imatinib resistance through the cytokine-cytokine receptor signalling pathway, the JAK-stat pathway, and the NF-KB signalling pathway. Indirubin and its derivatives may be new drugs that can combat imatinib resistance.

Keywords: Indirubin, Derivatives, Imatinib resistance, Drug target prediction, Gene microarray analysis, Protein network construction

Background

Chronic myeloid leukaemia (CML) is a clonal haematopoietic stem cell proliferation-induced myeloproliferative disease [1]. Because of its high heterogeneity and distinct molecular genetic features, it has attracted extensive attention from researchers. The unique cytogenetic features of CML include the Philadelphia chromosome t (9; 22)(q34; q11), forming a BCR-ABL fusion gene; this gene complex encodes a constitutively active form of the BCR–ABL fusion tyrosine kinase protein. The active site of the tyrosine kinase has a binding site for ATP [2]. Most signalling pathways activated by BCR-ABL are involved in promoting the development of cancer in bone marrow cells, including the Ras-MAPK pathway, the Src-Pax-Fak-Rac pathway, the phosphoinositide-3 kinase (PI3K)–Akt pathway, and the JAK-STAT pathway [3–6].

The development of the tyrosine kinase inhibitor (TKI) imatinib represents a milestone in CML treatment. Imatinib binds specifically to the ATP-binding site...
of BCR-ABL to form a fusion protein complex, locking in the active site [7]. This blocks CML cells whose active sites limit repeated cell growth and cell proliferation, killing the cancer cells. However, TKI treatment is long-term and induces resistance to TKI, often leading to poor clinical outcomes in CML patients. Drug resistance to TKIs is currently a clinical problem for CML. It is very important to find a strategy to inhibit imatinib resistance.

Classical traditional Chinese medicine (TCM) in China has been used for thousands of years. Especially in recent years, Chinese medicine has made some progress in the treatment of cancer. For example, Bu-Zhong-Yi-Qi-Decoction (BZYQD) has been reported to induce gastric cancer cell death by nonapoptotic mechanisms and to induce human ovarian cancer cell death by apoptotic mechanisms [8, 9]. Yu Ning, et al., through the combination BZYQD with cisplatin in cisplatin-resistant A549/DDP cells, showed that BZYQD exhibited direct cytotoxic and chemosensitising effects, suggesting that cotreatment with BZYQD and cisplatin might reverse cisplatin resistance by inducing ROS accumulation, activating apoptosis and autophagy by oxidative stress [10].

It was reported that Qingdai acted on a variety of pathways for the treatment of chronic myeloid leukaemia, including cytokine-cytokine receptor interaction, cell cycle, p53 signalling pathway, MAPK signalling pathway, and immune system-related pathways [11]. Indirubin is the most important and valuable compound in Qingdai; it has been determined to be the quality marker of Qingdai in the Chinese Pharmacopoeia (the State Pharmacopoeia Commission of China, 2015). Studies showed that indirubin and its derivatives inhibited imatinib resistance. For example, the AGM130 compound, derived from indirubin, known as a cyclin-dependent kinase inhibitor, was a strong candidate for treating imatinib-resistant CML [12]. Therefore, in this study, we will use the strategy of ’Drug Target Prediction-Gene Microarray Analysis-Protein Network Construction’ to explore the mechanism of indirubin and its derivatives in inhibiting imatinib resistance.

Data preparation
Identify indirubin and its derivatives
We identified indirubin and its derivatives from two sources: first by searching the PubChem database and then by manually searching PubMed to augment the data. PubChem (https://pubchem.ncbi.nlm.nih.gov) is a public repository for information on chemical substances and their biological activities. As of September 2015, it contained more than 157 million depositor-provided chemical substance descriptions, 60 million unique chemical structures and 1 million biological assay descriptions, covering approximately 10 thousand unique protein target sequences [13]. We searched the PubChem database with “indirubin” as the key word to identify indirubin and its derivatives, downloaded the compound 2D structures and finally downloaded the “smile” format. In order to increase the comprehensiveness of the data, we manually searched the relevant literature in the PubMed database for titles dealing with indirubin derivatives.

Identify the putative target of indirubin and its derivatives
It requires much manpower, material and financial resources to Identify targets of indirubin and its derivatives through experimentation. Therefore, we used a computerized virtual platform to screen for targets and then validated the targets by molecular docking or experimental verification. Swiss Target Prediction (http://www.swisstargetprediction.ch/), a web server to accurately predict the targets of bioactive molecules based on a combination of 2D and 3D similarity measures with known ligands, was used to predict the putative targets of the indirubin and its derivatives. Predictions can be carried out in five different organisms, and mapping predictions by homology within and between different species is enabled for close paralogs and orthologs [14]. The “smiles” formats of indirubin and its derivatives were imported into Swiss Target Prediction to predict their putative targets of action. It is noteworthy that the predicted putative target was limited to Homo sapiens. In order to improve the reliability of predictions, only high-probability targets were selected. All putative targets Identified were sent to the Therapeutic Target Database (TTD) (http://bidd.nus.edu.sg/group/cjttd/, 2015-09-10), the Comparative Toxicogenomics Database (CTD) (http://ctdbase.org/, 2017-12-05) and the PharmGKB (https://www.pharmgkb.org/) to verify whether these putative targets had some connection to CML.

Identify imatinib resistance related genes
Gene expression profiling analysis is a useful method with broad clinical application in the identification of tumour-related genes in various types of cancer, from molecular diagnosis to pathological classification, from therapeutic evaluation to prognosis prediction, and from

Methods
To decipher the mechanisms by which indirubin and its derivatives reverse imatinib resistance, we adopted the following strategies: (1) we identified the 2D structure of indirubin and its derivatives through data mining; (2) we downloaded GSE2810 from the GEO database and identified imatinib-resistant DEGs; (3) we predicted targets of indirubin and its derivatives using related databases; (4) we analysed the possible molecular mechanisms of indirubin and its derivatives reversing imatinib resistance; and (5) we verified the results through computer network molecular docking technology.
drug sensitivity to neoplasm recurrence [15]. Gene expression profile GSE2810 was downloaded from the Gene Expression Omnibus (GEO) database, GSE2810 data is based on the GPL2531 (Novusgene type 3 Hematology/Oncology TMU 667 array) platform, including 4 samples (2 imatinib-resistant samples and 2 imatinib-sensitive samples). It was submitted by Ohyashiki JH [16]. Quality control of gene expression data was performed using gene-specific probes. The analysis was carried out by using GEO2R, an online analysis tool for the GEO database, based on R language. We applied the analysis to classify the sample into two groups that had similar expression patterns in imatinib-sensitive and imatinib-resistant. We defined genes as differentially expressed (DEGs) when logFC > 1 or logFC < −1 (FC: Fold Change, the difference in the amount of gene expression in the sample). A p value < 0.05 was considered statistically significant. To further study the characteristics of DEGs and their functions, we analysed the DEGs with Gene Ontology and KEGG Pathway. Gene Ontology annotates and classifies genes by Molecular Function (MF), biological process (BP) and cellular component (CC). The p value of the GO term of the DEGs was calculated, and the most likely related GO term of the differential gene was located [17]. KEGG is an online biochemical energy database that contains a set of genomic and enzymatic methods and is an information resource for the systematic analysis of gene functions and associated high-level genomic functions [18]. ClueGo, a plugin for Cytoscape 3.5.1 software, provides systematic and comprehensive biologically functional annotation of high-throughput gene expression [19]. Therefore, ClueGo online tools were employed for GO and KEGG pathway analysis. P < 0.05 was considered significant.

Network construction
Protein-protein network (P-P network). P-P network was built using the relationship between the putative targets of Indirubin and its derivatives and Imatinib resistance related DEGs.

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

Analysis the protein-protein network
If the degree of a node is more than 2 fold of the median degree of all nodes in a network, such gene hub is believed to play a critical role in the network, and we treat it as major hub. The topological features of the target-target network are analysed by several important topological properties such as degree (the number of links to node) [20], betweenness (the number of shortest paths between pairs of nodes which run through node).
Molecular docking simulation

Using computer molecular docking simulation techniques, we evaluated the credibility of the findings. SystemsDOCK (http://systemsdock.unit.oist.jp/) was utilized for molecular docking. SystemsDock is a web server for network pharmacology-based prediction and analysis, which allows docking simulation and molecular pathway mapping for comprehensive characterization of ligand selectivity and interpretation of ligand action on a complex molecular network. The score reported by docK-IN is a negative logarithm of the experimental dissociation/inhibition constant, typically ranging from 0 to 10 (indicating weak to strong binding). We conducted molecular docking between the small molecule compounds and their putative targets to assess the inhibitory potential of indirubin and its derivatives in imatinib resistance.

Result

Data preparation

**Indirubin and 41 derivatives and putative targets**

We identified indirubin and 41 derivatives from the database and downloaded “smiles” format and 2D structures. The putative targets of indirubin and its derivatives were predicted based on structural similarities. Indirubin and 41 derivatives and putative targets are listed in Table 1.

**Imatinib resistance related genes**

After gene chip data analysis, we obtained a heat map of the differentially expressed genes of the gene chip G2810 (Additional file 1: Fig. S1), and a P-P network about DEGs was constructed. The red nodes represent up-regulated differentially expressed genes, and the blue nodes represent down-regulated differentially expressed genes.

![Fig. 1](image_url)

**Fig. 1** Based on GEO2R analysis, differentially expressed genes of imatinib resistance in chronic myeloid leukemia were identified from GEO2810 (logFC > 1 or logFC < −1; P < 0.05), and a P-P network about DEGs was constructed. The red nodes represent up-regulated differentially expressed genes, and the blue nodes represent down-regulated differentially expressed genes.
125 DEGs with imatinib resistance (Fig. 1), of which 66 were up-regulated and 59 were down-regulated. According to FC, the top 10 significantly up-regulated DEGs and down-regulated DEGs are shown in Table 2. Go analysis and KEGG analysis of DEGs, we found that DEGs of imatinib resistance were closely related to biological processes including immune responses, regulation of protein modification process, regulation of phosphorylation, and regulation of cellular protein metabolic processes. DEGs were mainly involved in cytokine-cytokine receptor interaction pathways.

CCL13, the first significantly up-regulated chemokine, is a chemotactic factor that attracts monocytes, lymphocytes, basophils and eosinophils [24]. MAPK11, the second significantly up-regulated chemokine, plays an important role in the cascades of cellular responses evoked by extracellular stimuli, including proinflammatory cytokines and physical stress leading to direct activation of transcription factors. The study of Huang J et al. showed that the ERK signalling pathway was more activated in epirubicin treated triple-negative breast cancer (TNBC), possibly contributing to epirubicin resistance, suggesting that the ERK pathway could be used as a novel candidate for targeting therapy in refractory and relapse TNBC [25]. MLH1, the first significantly down-regulated DEG, has been shown to play an important role in haematologic malignancies. The novel mutation was also revealed to be a somatic aberration occurring prior to the initiation of the blast phase in a chronic myelogenous leukaemia (CML) patient. Among the possible MLH1 partners involved in signalling MMR or apoptosis is the proto-oncogene c-MYC, closely associated with cellular proliferation [26]. BCL10, the second significantly down-regulated chemokine, was involved in adaptive immune responses. Proliferation of NIK and IKK cells is promoted by pro-caspase-9 maturation and NF-κB activation.

To further explain the function of differentially expressed genes, we performed functional enrichment analysis of all differential genes based on GO analysis, and performed passway enrichment analysis of all differential genes based on KEGG analysis. We chose significantly up-regulated and down-regulated GO categories based on functional enrichment. The analysis results are shown in Figs. 2 and 3. Through GO analysis, we reached the following conclusions: up-regulated differentially expressed genes were primarily involved in the regulation of cell apoptosis, including immune responses, regulation of apoptosis, regulation of programmed cell death, regulation of cell death, regulation of transcription, cell death, death and DNA binding. The down-regulated DEGs were primarily related to cellular structures, such as cytoplasm, nucleus, extracellular space, positive regulation of transcription from the RNA

| Group               | Genesymbol | Gene Description                  | Fold Change |
|---------------------|------------|-----------------------------------|-------------|
| Upregulated genes   | CCL13      | C-C motif chemokine 13            | 9.39035     |
|                     | MAPK11     | Mitogen-activated protein kinase 11| 7.52975     |
|                     | PDCD4      | Programmed cell death protein 4   | 7.43475     |
|                     | BCL2       | Bcl2-associated agonist of cell death| 7.22081     |
|                     | CCL27      | C-C motif chemokine 27            | 6.79919     |
|                     | TCEB3B     | transcription elongation factor B subunit 3B | 6.65061 |
|                     | ANAPC10    | anaphase promoting complex subunit 10 | 6.21695 |
|                     | IL1R1      | interleukin 1 receptor type 1     | 6.14025     |
|                     | TCF4       | transcription factor 4             | 5.79877     |
|                     | TFAP2A     | transcription factor AP-2 alpha    | 5.65156     |
| Downregulated genes | MLH1       | MutL homolog 1                     | −10.7446    |
|                     | BCL10      | B-cell CLL/lymphoma 10             | −8.27759    |
|                     | MAP3K4     | mitogen-activated protein kinase kinase 4 | −8.1475 |
|                     | CDK9       | cyclin dependent kinase 9          | −6.66841    |
|                     | APO8       | apolipoprotein B                   | −6.5818     |
|                     | PDGFC      | platelet derived growth factor C    | −6.26762    |
|                     | IL10RA     | interleukin 10 receptor subunit alpha | −5.64569 |
|                     | IL12A      | interleukin 12A                    | −5.49548    |
|                     | CDC14A     | cell division cycle 14A            | −5.20635    |
|                     | ALOX5      | arachidonate 5-lipoxygenase        | −5.20383    |
polymerase II promoter, transcription factor activity and sequence-specific DNA binding growth factor activity. We performed pathway enrichment analysis of differentially expressed genes to identify the biological pathways. Up-regulated differentially expressed genes were primarily involved in cytokine-cytokine receptor interaction, chemokine signalling pathways, the Toll-like receptor signalling pathway, the neurotrophin signalling pathway, leukocyte transendothelial migration, the MAPK signalling pathway, haematopoietic cell lineage, apoptosis, the T cell receptor signalling pathway and the JAK-STAT signalling pathway. Pathways dramatically altered among down-regulated genes were the cytokine-cytokine receptor interaction, Toll-like receptor signalling pathway, Jak-STAT signalling pathway, pathways in cancer, the NOD-like receptor signalling pathway, apoptosis, cell cycle and the p53 signalling pathway.

To identify the relationship between the putative targets of indirubin and its derivatives and DEGs of imatinib resistance, we constructed a P-P network of putative targets and DEGs (Fig. 4). The T-T network consisted of 171 nodes and 1082 edges. The major hubs in the hub interaction network were determined by calculating four features: degree, betweenness, closeness and K-coreness. We showed the major hubs in Fig. 3. After screening, we identified a total of 62 major hubs (Table 3), including 11 (EGFR, JAK2, ERBB2, CHUK, CDK5, KIF11, DRD2, CDK3, HTR1A, JAK3 and TYK2) indirubin and derivative targets and 51 DEGs for imatinib resistance. These 11 major hubs were closely related to DEGs that were
resistant to imatinib. Indirubin and its derivatives may inhibit imatinib resistance through the regulation of these genes.

We manually screened out small molecule compounds that affected 11 major hubs in the putative target. After screening, a total of 15 small molecule compounds affected these putative targets, including 1, 3, 4, 5, 6, 8, 11, 14, 21, 24, 26, 33, 36, 40, 41. These derivatives may all inhibit imatinib resistance. To further verify this conclusion, we evaluated docking of small molecule compounds and their putative targets that were included in the major hubs. The docking results are shown in Table 4.

Discussion
Qingdai is a traditional Chinese medicine used to treat CML; it is the major active TCM of Qing-Huang-San [27], a Chinese traditional medicine used for the treatment of CML symptoms. It has been widely used in China and has achieved good clinical results. Indirubin is the major active component of Qingdai. Numerous studies have shown that indirubin and its derivatives not only promote apoptosis of CML cells but also inhibit imatinib resistance, including indirubin, indirubin derivative E804, and indirubin-3-acetoxime [28–30]. The exact mechanism of action remains unclear. Therefore, we used the Drug Target Prediction-Gene Microarray Analysis-Protein Network Construction model to investigate the mechanism by which indirubin and its derivatives inhibit imatinib resistance. Various methods, including indirubin derivative screening, drug target search screening, gene chip analysis, network construction, network target analysis, and molecular docking were combined to perform this study. A total of 42 small-molecule compounds were collected and predicted for putative targets. A total of 125 DEGs were selected for imatinib resistance. A total of 15 small-molecule compounds were found to inhibit imatinib resistance by 11 related genes. In our research, data mining of existing databases allows for the objective and
rapid discovery of associations and identification of potential drug targets to facilitate the discovery of drugs that inhibit imatinib resistance.

CML is a major haematological malignancy. Imatinib is one of the primary drugs for the treatment of chronic myelogenous leukaemia; however, due to the resistance to imatinib, we were forced to study new drugs to inhibit the resistance to imatinib [31]. Drug resistance involves multiple steps and multiple genes. Therefore, various studies have analysed the differences in gene expression in imatinib-resistant and non-resistant genes by genomic microarrays. In the present study, we performed Go analysis and KEGG analysis on 125 differentially expressed genes and found that the resistance to imatinib was closely related to the following signalling pathways: (1) cell cycle, cell transcription, proliferation, apoptosis, and angiogenesis-related pathways; (2) cytokine-cytokine receptor interaction and chemokine signalling pathways; (3) cancer system related pathways, including pathways in cancer, the p53 signalling pathway and Jak-STAT signalling; (4) the immune system signalling pathway, the T cell receptor signalling pathway, the Toll-like receptor signalling pathway and the NOD-like receptor signalling pathway.

By analysing DEGs, we found that individual genes can serve as biomarkers for imatinib resistance. In up-regulated DEGs, CCL-13, the most significant up-regulated DEGs, is a chemokine that induces eosinophilic chemicals [32]; it can be involved in the interaction between haematopoietic stem cells and the bone marrow microenvironment [33]. In addition, the cytokine-cytokine receptor and chemokine signalling pathways involved in CCL-13 are important pathways involved in imatinib resistance. MAPK11 is the second most prominently expressed gene in the up-regulated differentially expressed genes for imatinib resistance, and MAPK11 is an important constituent gene of the MAPK signalling pathway and is involved in the regulation of various angiogenesis-related diseases [34]. The MAPK signalling pathway is significantly augmented after imatinib resistance and may be closely related to imatinib resistance. MAPK11 is also involved in up-regulating multiple regulatory pathways for DEGs, including the Toll-like receptor signalling pathway and leukocyte transendothelial migration. PIK3CD is involved in almost all pathways involved in the up-regulation of differentially expressed genes and is significantly augmented in the course of imatinib resistance.

Mesenchymal stem cells (MSC) from BM of chronic myeloid leukaemia (CML) patients on interaction with CML cells or its secreted factors, secreted high levels of IL6, providing a survival advantage to CML cells from imatinib-induced apoptosis [35]; Thus, IL6 may contribute to CML immune escape. Moreover, IL6 is involved in the cytokine-cytokine receptor interaction, the Jak-STAT signalling pathway, and pathways in cancer; therefore, it is closely related to imatinib resistance.

In the down-regulated DEGs, CASP8, an apoptosis-related factor, is an important apoptosis-related gene. Investigators used quantitative PCR to study apoptotic...
| ID | Major target                                           | UniProt ID | Gene name   |
|----|-------------------------------------------------------|------------|-------------|
| MT1| Interleukin-6                                         | P05231     | IL6         |
| MT2| Epidermal growth factor receptor                      | P00533     | EGF         |
| MT3| Transcription factor AP-1                            | P05412     | JUN         |
| MT4| Apoptosis regulator Bcl-2                            | P10415     | BCL2        |
| MT5| Heat shock protein HSP 90-alpha                       | P07900     | HSP90AA1    |
| MT6| Serine-protein kinase ATM                             | Q13315     | ATM         |
| MT7| Tyrosine-protein kinase JAK2                          | O60674     | JAK2        |
| MT8| Phosphatidylinositol 4,5-bisphosphate 3-kinase catalytic subunit delta isoform | O00329     | PIK3CD      |
| MT9| Receptor tyrosine-protein kinase erbB-2               | P04626     | ERBB2       |
| MT10| Baculoviral IAP repeat-containing protein 5          | O15392     | BIRC5       |
| MT11| Interleukin-1 beta                                    | P01584     | IL1B        |
| MT12| Receptor-type tyrosine-protein phosphatase C         | P08575     | PTEN        |
| MT13| Mitogen-activated protein kinase 11                   | Q15759     | MAPK11      |
| MT14| Interleukin-10                                        | P22301     | IL10        |
| MT15| C-X-C chemokine receptor type 4                       | P61073     | CXCR4       |
| MT16| Amyloid-beta A4 protein                               | P05067     | APP         |
| MT17| Inhibitor of nuclear factor kappa-B kinase subunit alpha | O15111     | CHUK        |
| MT18| POU domain, class 5, transcription factor 1          | Q01860     | POU5F1      |
| MT19| Cyclin-dependent-like kinase 5                        | Q00535     | CDK5        |
| MT20| ATP-binding cassette sub-family G member 2           | Q9UNQ0     | ABCG2       |
| MT21| Cation-independent mannose-6-phosphate receptor       | P11717     | IGF2R       |
| MT22| Cyclin-dependent kinase inhibitor 1B                  | P46527     | CDKN1B      |
| MT23| ALK tyrosine kinase receptor                           | Q9UM73     | ALK         |
| MT24| E3 ubiquitin-protein ligase CBL                       | P22681     | CBL         |
| MT25| Substance-P receptor                                  | P25103     | TACR1       |
| MT26| Wilms tumor protein                                   | P19544     | WT1         |
| MT27| ETS-related transcription factor Elf-3                | P78545     | ELF3        |
| MT28| G1/S-specific cyclin-D2                               | P30279     | CCND2       |
| MT29| Amine oxidase (flavin-containing) A                    | P21397     | MAOA        |
| MT30| Metalloproteinase inhibitor 1                         | P20414     | TiMP1       |
| MT31| Kinesin-like protein KIF11                            | P52732     | KIF11       |
| MT32| Cell division cycle protein 16 homolog                | Q13042     | CDC16       |
| MT33| Nitric oxide synthase, brain                          | P29475     | NOS1        |
| MT34| DNA (cytosine-5)-methyltransferase 3B                 | Q9UBC3     | DNMT3B      |
| MT35| 1-phosphatidylinositol 4,5-bisphosphate phosphodiesterase gamma-1 | P19174     | PLCG1       |
| MT36| POU domain class 2-associating factor 1               | Q16633     | POU2AF1     |
| MT37| E3 ubiquitin-protein ligase XIAP                      | P98170     | XIAP        |
| MT38| Anaphase-promoting complex subunit 10                 | Q9UM13     | ANAPC10     |
| MT39| Runt-related transcription factor 1                   | Q01196     | RUNX1       |
| MT40| WD repeat and FYVE domain-containing protein 2        | Q96PS3     | WDF5Y2      |
| MT41| M-phase inducer phosphatase 1                         | P30304     | CDC25A      |
| MT42| D(2) dopamine receptor                                | P14416     | DRD2        |
| MT43| CASP8 and FADD-like apoptosis regulator               | Q15519     | CASP8       |
| MT44| Cyclin-dependent kinase 3                             | Q00526     | CDK3        |
gene expression profile before and after imatinib treatment; they suggested that apoptosis-related gene expression profiles were associated with primary resistance to imatinib [36]. IL12A enhances cellular immunity in the treatment of CML. Studies have shown that immunotherapy enhanced the efficacy of imatinib, and low expression of IL12A led to immune escape of CML cells [37]. Therefore, CCL13, MAPK11, PIK3CD, IL6, CASP8, and IL12A play an important role in the process of imatinib resistance and can be used as biomarkers for imatinib resistance.

To elucidate the relationship between indirubin and its derivatives and imatinib resistance, we constructed a P-P network [38]. By analysing the P-P network, we found that there was a close relationship between the putative target of indirubin and its derivatives and DEGs of imatinib resistance. Through screening, we characterised a total of 11 putative targets [39]. Indirubin and its derivatives may inhibit imatinib resistance through these 11 putative targets. Based on 11 putative targets, we screened 15 small molecule compounds.

Among the 11 putative targets, gefitinib, an EGFR inhibitor, was tested in combination with imatinib in K562 CML cell line using MTT cell proliferation assay and was found to have a synergistic antiproliferative activity; EGFR inhibits or reverses imatinib resistance by enhancing the ability of imatinib to bind at the ATP-binding site of Bcr-Abl kinase [40]. The study found that JAK2 and JAK3 had antiproliferative effects on imatinib-resistant BCR-ABL(+) cells [41], and the administration of imatinib plus a JAK inhibitor reduced expression of stem cells markers, enhancing the antitumour effects of imatinib in CML cells [42]. Human ERBB2 is a proto-oncogene that codes for the erbB-2 epithelial growth factor receptor [43]. CHUK plays an important role in the NF-κB signalling pathway; indirubin and its derivatives inhibited CML cell proliferation by inhibiting CHUK activation of the NF-κB signalling pathway [44]. A study showed that NF-κB represents a potential target for molecular therapies in CML [45]. KIF11 inhibited cell proliferation by blocking the cycle of CML cells. The data showed that KIF11 was overexpressed in BCR-ABL+ CML cells and may become a novel treatment agent for patients with CML [46]. Administration of the imatinib plus JAK inhibitor reduces the expression of stem cell markers, such as ABCG2 and ALDH1A. Blocking JAK3 with imatinib and JAK3 inhibitors may represent a new therapeutic strategy for eradicating LSCs and preventing CML recurrence [47].

We identified a total of 15 small-molecule compounds that showed potential inhibition or reversal of resistance to imatinib. Active indirubins might inhibit T315I Abl kinase through unprecedented binding to both active and Src-like inactive conformations [30]. The AGM130 compound is derived from indirubin; data showed that the AGM130 compound efficiently decreased the viability of CML-derived K562 cells. Moreover, this compound also efficiently decreased the viability of imatinib-resistant

### Table 3 The 62 major targets information of P-P network (Continued)

| ID  | Major target                                      | Uniprot ID | Gene name |
|-----|---------------------------------------------------|------------|-----------|
| MT45| Tyrosine-protein phosphatase non-receptor type 2  | P17706     | PTPN2     |
| MT46| DNA mismatch repair protein Mlh1                  | P40692     | MLH1      |
| MT47| Wee1-like protein kinase                          | P30291     | WE1       |
| MT48| Neural cell adhesion molecule 1                   | P30291     | NCAM1     |
| MT49| Caspase-9                                         | P52211     | CASP9     |
| MT50| Toll-like receptor 3                              | O15455     | TLR3      |
| MT51| C-X-C motif chemokine 2                           | P19875     | CXCL2     |
| MT52| 5-hydroxytryptamine receptor 1A                    | P08908     | HTR1A     |
| MT53| Mothers against decapentaplegic homolog 7         | O15105     | SMAD7     |
| MT54| Transcription factor 4                            | P15884     | TCF4      |
| MT55| Tyrosine-protein kinase JAK3                       | P52333     | JAK3      |
| MT56| Interleukin-2 receptor subunit alpha              | P01589     | IL2RA     |
| MT57| Non-receptor tyrosine-protein kinase TYK2         | P29597     | TYK2      |
| MT58| Dual specificity protein phosphatase CDC14A       | Q9UNH5     | CDC14A    |
| MT59| Cyclin-dependent kinase 9                         | P50750     | CDK9      |
| MT60| Presenilin-1                                      | P49768     | PSEN1     |
| MT61| Apolipoprotein B-100                              | P04114     | APOB      |
| MT62| C-X-C motif chemokine 13                           | O43927     | CXCL13    |
Table 4 The docking results of molecule compounds and their putative targets. '4 + EGFR' represents the molecular docking of the indirubin derivative numbered 4 with EGFR, and Score represents the score identified by molecular docking.

| Molecule | Score |
|----------|-------|
| 4 + EGFR | 5.635 |
| 3 + JAK2 | 4.542 |
| 8 + JAK2 | 4.326 |
| 36 + AK2 | 4.871 |
| 14 + ERBB2 | 4.114 |
| 35 + CHUK | 4.023 |
| 1 + JAK2 | 6.956 |
| 1 + CDK5 | 7.002 |
| 5 + CDK5 | 7.137 |
| 5 + JAK2 | 6.883 |
| 6 + CDK5 | 4.898 |
| 6 + JAK2 | 4.764 |
| 11 + CDK5 | 6.783 |
| 41 + KIF11 | 4.007 |
| 40 + DRD2 | 4.446 |
| 33 + DRD2 | 4.471 |
| 21 + CDK3 | 4.487 |
| 24 + CDK3 | 5.534 |
| 26 + CDK3 | 4.009 |
| 41 + HTR1A | 4.243 |
| 3 + JAK3 | 4.683 |
| 36 + TYK2 | 4.205 |
CML cells in in vitro and in vivo systems [5]. E804, the most potent in indirubin derivative, blocked Stat5 signaling in human K562 CML cells, inhibiting the SFK/Stat5 signaling pathway downstream of Bcr-Abl, leading to apoptosis of K562, KCL-22 M and primary CML cells [48]. In the present study, we identified small-molecule compounds of indirubin and its derivatives that could potentially inhibit imatinib resistance through drug target prediction, gene microarray analysis, and network construction, accelerating the discovery of new drugs for the treatment of imatinib resistance.

Finally, we used computer simulation techniques to dock selected small-molecule compounds to putative targets, and docking scores showed meaningful results, indicating that our series of strategies can achieve the desired results.

Conclusion
Definition of a potential drug target is an important first step in the process of drug discovery and drug design. Gene microarray analysis and protein network mapping can be key tools for identification of the factors that play a role in disease progression and thus are the potential drug targets. Subsequently, molecular docking experiments in silico can be used to predict putative interaction of small molecule compounds with the identified targets. In this study, based on the above methods, the mechanism of action of indirubin and its derivatives in inhibiting or reversing the resistance to imatinib was explored, and biomarkers and novel therapeutic targets that inhibited the resistance to imatinib were discovered. We validated experimental results by computerized molecular docking techniques. A limitation of this study was that the results were initially verified by computer simulation, and further verification can be achieved through experimental research.

Additional file

Additional file 1: Figure S1. Heat maps of differentially expressed genes associated with imatinib resistance (we selected 100 genes with the most significant differential expression) (**P < 0.05**). The color from blue to red shows a trend from low to high expression. (JPG 298 kb)

References
1. Ostorrio S, Escudero-Vlaplana V, Gómez-Centurión I, González-Arias E, García-González X, Diez JL. Inadequate response to imatinib treatment in chronic myeloid leukemia due to a drug interaction with phenytoin. J Oncol Pharm Pract. 2017;10:1078155217743565.
2. Kidan N, Khamaiseh H, Ruimi N, Rötman S, Estel E, Dally N. Ectopic Expression of Snail and Twist in Ph+ Leukemia Cells Uregulates CD44 Expression and Alters Their Differentiation Potential. J Cancer. 2017;8:2952–68.
3. Hilger RA, Scheulen ME, Strumborg D. The Ras-Raf-MEK-ERK pathway in the treatment of cancer. Onkologie. 2002;25:511–8.
4. Bunnidge K, Wennerberg K, Rho and Rac take center stage. Cell. 2004;116:167–79.
5. Bratton MR, Duong BN, Elliott S, Weldon CB, Beckman BS, McLachlan JA. Regulation of ERα mediated transcription of Bcl-2 by PI3K-Akt crosstalk: implications for breast cancer cell survival. Int J Oncol. 2010;37:541–50.
6. Constantinides SN, Girardot M, Peccquet C.Mining for JAK-STAT mutations in cancer. Trends Biochem Sci. 2008;33:122–31.
7. Eck MJ, Manley PW. The interplay of structural information and functional studies in kinase drug design, insights from BCR-ABL. Curr Opin Cell Biol. 2009;21:288–95.
8. Chao T, Fu P, Chang C, Chang S, Wao FC, Lin C. Prescription patterns of Chinese herbal products for post-surgery colon cancer patients in Taiwan. J Ethnopharmacol. 2014;155:702–8.
9. Zhu K, Fukasawa I, Furuno M, Inaba F, Yamazaki T, Kamemori T. Inhibitory effects of herbal drugs on the growth of human ovarian cancer cell lines through the induction of apoptosis. Gynecol Oncol. 2004;97:405–9.
10. Yu N, Xiong Y, Wang C, Bu-Zhong-Yi-Qi Decoction, the water extract of chinese traditional herbal medicine, enhances cisplatin cytotoxicity in A549/DDP cells through induction of apoptosis and autophagy. Biomed Res Int. 2017.
11. Li HY, Liu LJ, Liu C, et al. Deciphering key pharmacological pathways of Qingdaiding acting on chronic myeloid leukemia using a network pharmacology-based strategy. Med Sci Monit. 2018;24:5668–88.
12. Kim WS, Lee MJ, Kim DH, Lee JE, Kim J, Kim YC. S-OH-S-nitro-Indirubin oxime (AGM130), an Indirubin derivative, induces apoptosis of Imatinib-resistant chronic myeloid leukemia cells. Leuk Res. 2013;37:427–33.

13. Kim S, Thiessen PA, Bolton EE, Chen J, Fu G, Gindulyte A, Han L, He J, He S, Shoemaker BA, Wang J, Yu B, Zhang J, Bryant SH. PubChem substance and compound databases. Nucleic Acids Res. 2016;44:D202–13.

14. David G, Aurelien G, Matthias W, Antoine D, Olivier M, Vincent Z. AntisTargetPrediction: a web server for target prediction of bioactive small molecules. Nucleic Acids Res. 2014;42:32–8.

15. Freije WA, Castro-Vargas FE, Fang Z, Horvath S, Cloughesy T, Liu AM. Gene expression profiling of gliomas strongly predicts survival. Cancer Res. 2004;64:5659–10.

16. Nunoda K, Tauchi T, Takaku T, Obake S et al. Identification and functional signature of genes regulated by structurally different ABL kinase inhibitors. Oncogene. 2007;26:4179–88.

17. Gene Ontology Consortium. The gene ontology (GO) project in 2006. Nucleic Acids Res. 2006;34:D322–6.

18. Kaneshita M, Goto S. KEGG: Kyoto encyclopedia of genes and genomes. Nucleic Acids Res. 2000;28:O27–30.

19. Paul S, Andrew M, Owen J, et al. Cytoescape: a software environment for integrated models of biomolecular interaction networks. Genome Res. 2003;13:2498–504.

20. Zhang YQ, Guo QY, Li QY, et al. Main active constituent identification in Guanxinjia capsule, a traditional Chinese medicine, for the treatment of coronary heart disease complicated with depression. Acta Pharmacol Sin. 2018;39:975–87.

21. Stefan W, Eivind A. Evolutionary cores of domain co-occurrence networks. BMC Evol Biol. 2005;5:24.

22. Wang Y, Li L, Li C. Drug target prediction based on the herbs components: the study on the multitargets pharmacological mechanism of Qishenki acting on the coronary heart disease. Evid Based Complement Alternat Med. 2012.

23. Han KY, Matsuka Y, Asai Y, Kamiyoshi K, Watanabe T, Kawaoka Y. SystemsDock: a web server for network pharmacology-based prediction and analysis. Nucleic Acids Res. 2016;44:4507–13.

24. Cossio-Ayala M, Domínguez-López M, Mendez-Enriquez E, Portillo-Tellez MDC, García-Hernández E. In vitro and in vivo antimicrobial activity of a synthetic peptide derived from the C-terminal region of human chemokine CCL13 against Pseudomonas aeruginosa. Peptides. 2017;94:49–55.

25. Huang J, Luo Q, Xiao Y, Li H, Kong L, Ren G. The implication from RAS/RAF/ERK signaling pathway increased activation in epirubicin treated triple negative breast cancer. Oncotarget. 2017;8:108294–90.

26. Amikam D, Leshanski S, Sagi M. A novel MLH1 mutation harbored as a germ line aberration by a young woman of an HNPCC-like family and exhibited by a CML patient when occurring prior to the initiation of the blast phase concomitant with a C-MYC amplification. Int J Mol Med. 2006;17:1023–6.

27. Liu C, Liu L, ZHOU C, ZHUANG J, LIU H, SUN CG. Analysis of mechanism of indigo Naturalis in treating chronic Myelocytic leukemia based on three-dimensional model of protein-protein interaction network-molecular docking technique-in vitro experiment. Chin J Experiment Trad Med Form. 2017;23:206–11.

28. Lee MY, Liu YW, Chen MH, Wu JY, Ho HY, Wang QF. Indirubin-3′-monoxime promotes autophagic and apoptotic death in JH11 human acute lymphoblastic leukemia cells and K562 human chronic myelogenous leukemia cells. Oncol Rep. 2013;29:2072–8.

29. Heshmati N, Wagner B, Cheng X, Scholz T, Kamsy M, Eisenbrand G. Physicochemical characterization and in vitro permeation of an indirubin derivative. Eur J Pharm Sci. 2013;50:467–75.

30. Gaboriaud-Kolar N, Myrianthopoulos V, Vougogiannopoulou K, Geralyomatos P, Home DA, Jove R. Natural-based Indirubins display potent cytotoxicity toward wild-type and T315I-resistant leukemia cell lines. J Nat Prod. 2016;79:2464–71.

31. Larocque EA, Naganna N, Opoku-Temeng C, Lambrech AM, Sintim HO. Alkylresorcinamide-Based Compounds as ABL1 Inhibitors with Potent Activities against Drug-Resistant CML-T harboring ABL1(T315I) Mutant Kinase. ChemMedChem. 2018; 12:1172-80.

32. Yang T, Li Y, Lyu Z, Huang K, Corrigan CJ, Ying S. Characteristics of Proinflammatory Cytokines and Chemokines in Airways of Asthmatics: Relationships with Disease Severity and Infiltration of Inflammatory Cells. Chin Med J (Engl). 2017;130:2033–40.

33. Mukaida N, Tanabe Y, Baba T. Chemokines as a conductor of bone marrow microenvironment in chronic myeloid leukemia. Int J Mol Sci. 2017;18:1824.

34. Chun YX, Hui ZD, Boud LW, Qiang UK, Yu TJ, Chao CZ, Liang MX, Hua LJ, Fan Q. IncRNA ENSMUST0000034285 Increases MAPK1 Activity, Regulating Aging-Related Myocardial Apoptosis. J Gerontol A Biol Sci Med Sci. 2018;73:1010-17.

35. Kumar A, Anand T, Bhattacharyya J, Sharma A, Jaganathan BG. K562 chronic myeloid leukemia cells modify osteogenic differentiation and gene expression of bone marrow stromal cells. J Cell Commun Signal. 2018;12:441-50.

36. Ferreira AF, Oliveira GL, Tognon R, Collassanti MD, Zanichelli MA, Hamerschlak N. Apoptosis-related gene expression profile in chronic myeloid leukemia patients after imatinib mesylate and dasatinib therapy. Acta Haematol. 2015;133:54–64.

37. Tao K, Li YS, Wang D, Qi JY, Deng YP, Wang HX. Enhancement of specific cellular immune response induced by glycoaryl-phosphatidylinositol-anchored CRAF/ABL and mll-12. Cancer Biol Ther. 2011;12:881–7.

38. Yu GH, Zhang Q, RWQ, Dong L, Li JF, Geng Y. Network pharmacology-based identification of key pharmacological pathways of Yin–Huang–Qing–Fei capsule acting on chronic bronchitis. Int J Chron Obstruct Pulmon Dis. 2016;12:885–94.

39. Yue SJ, Liu J, Feng WW, Zhang FL, Chen JX, Xin LT. System pharmacology-Based Dissection of the synergistic mechanism of Huangqi and Huanglian for diabetes mellitus. Front Pharmacol. 2017;8:694.

40. Singh YK, Chang HH, Kuo CC, Shiao HY, Hsieh HP, Courmar MS. Drug repurposing for chronic myeloid leukemia: in silico and in vitro investigation of Drugbank database for allosteric Bcr-Abl inhibitors. J Biomol Struct Dyn. 2017;35:1833–48.

41. Yagi K, Shimada A, Sendo T. Pharmacological inhibition of JAK3 enhances the antitumor activity of imatinib in human chronic myeloid leukemia. Eur J Pharmacol. 2018;825:28–33.

42. Tanaka R, Squires MS, Kimura S, Yokota A, Nagao R, Yamauchi T. Activity of the multitargeted kinase inhibitor, AT9283, in imatinib-resistant BCR-ABL-positive leukemic cells. Blood. 2010;116:2089–95.

43. Santos S, Baptista CS, Abreu RM, Bastos E, Amorim L, Gut MG. ERBB2 in cat mammary neoplasias disclosed a positive correlation between RNA and protein low expression levels: a model for erbB-2 negative human breast cancer. PLoS One. 2013;8:e83673.

44. Kirschner D, Duyster J, Ottmann O, Schmid RM, Bergmann L, Munzert G. Mechanisms of Bcr-Abl-mediated NF-kappaB/Rel activation. Exp Hematol. 2017;55:887-91.

45. Lu Y, Jamieson L, Brasier AR, Fields AP. NK-kappaB/Rel transactivation is required for atypical protein kinase C iota-mediated cell survival. Oncogene. 2001;20:4777–92.

46. Yin Y, Sun H, Xu J. Kinesin spindle protein inhibitor SB743921 induces mitotic arrest and apoptosis and overcomes imatinib resistance of chronic myeloid leukemia cells. Leuk Lymphoma. 2015;56:1813–20.

47. Kenta Y, Akira S, Toshiai S. Pharmacological inhibition of JAK3 enhances the antitumor activity of imatinib in human chronic myeloid leukemia. Eur J Pharmacol. 2018;825:28–33.

48. Nam S, Scuto A,Yang F, Chen W, Park S, Yoo HS. Indirubin derivatives induce apoptosis of chronic myelogenous leukemia cells involving inhibition of Stat5 signaling. Mol Oncol. 2012;6:276–83.