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Combination of *Gentiana rhodantha* and *Gerbera anandria* in the BL02 formula as therapeutics to non-small cell lung carcinoma acting via Rap1/cdc42 signaling: A transcriptomics/ bio-informatics biological validation approach

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ABSTRACT

Background: Non-small cell lung cancer (NSCLC) ranks the most commonly diagnosed and highest mortality-leading cancer worldwide despite a variety of treatment strategies are available. The highly heterogeneous and aggressive property of NSCLC as well as its poor prognosis indicates the need for novel therapeutic targets identification. The objective of this study is to identify potential targets from the adjuvant herbal formula BL02 using a combined approach of high throughput transcriptomics and network pharmacology.

Methods: The quality and stability of BL02 were assessed by UHPLC analysis. The inhibitory effect of BL02 on NSCLC was measured by *in vivo* orthotopic intrathoracic mouse model and *in vitro* cellular models. *EGFR*-mutant HCC827 and wild type A549 cell lines were employed. Transcriptomics analysis was introduced to profile the gene expression of NSCLC cells treated with BL02; Network pharmacology and molecular docking analyses predicted the interaction of compounds and NSCLC targets. Immuno-blotting and pull-down assays verified the putative targets.

Results: The UHPLC analysis revealed that BL02 was relatively stable between batches of production and for 24 months of storage. Orally administration of BL02 was safe and effective to inhibit pulmonary NSCLC growth in mice implanted with A549 and HCC827-generated tumors. BL02 exhibited relatively low cytotoxicity to NSCLC cells *in vitro*, but potently suppressed NSCLC cell motility. The transcriptomic analysis illustrated that EGFR and cellular adhesion-related signaling is involved in BL02 action. Further bioinformatics analysis validated BL02 activity is mediated by cdc42-regulated signaling. BL02 depolymerized the actin cytoskeleton through suppressing cdc42 and deactivating its upstream molecule Rap1. These effects may be primarily mediated by the direct binding of 5-methylcoumarin-4-cellobioside and mangiferin from BL02 to Rap1 protein.

Conclusion: Our study proposes an integration model of experimental, transcriptomic and bioinformatics analyses in the identification of novel therapeutic target of NSCLC from an adjuvant herbal formula BL02. Our findings revealed that inhibition of Rap1/cdc42 signaling by active compounds 5-methylcoumarin-4-cellobioside and mangiferin from BL02 might be potentially effective therapy for NSCLC.

1. Introduction

Lung cancer is the most commonly diagnosed carcinoma worldwide and associated with a high mortality rate. It was estimated that 18.4% of total cancer-leading death was attributable to lung cancer [1]. Non-small cell lung cancer (NSCLC) is the most prevalent type, which accounts for more than 85% of the total lung cancer incidence [2]. Depending on the stages when it first diagnosed, different types of modalities including surgery, chemotherapy, targeted therapies as well as immunotherapy are used either alone or in combination for NSCLC treatment. Owing to the high histologically heterogeneous property of NSCLC, molecularly targeted therapy has yielded some health benefit, especially to the advanced NSCLC patients who have limited treatment choices. Epidermal growth factor receptor gene (*EGFR*) mutation, for

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example, was detected in more than 60% of the NSCLC patients and these patients' subgroups were highly responsive to EGFR inhibitors such as gefitinib and erlotinib [3]. Nonetheless, *EGFR* mutation only showed higher frequency in Asian population relative to Caucasians (47.9% *vs*. 19.2%). Caucasians are more prone to KRAS mutation in which they are insensitive to EGFR inhibitor and the targeted therapeutics to date are still limited [4]. Therefore, the effort to seek for alternative or complementary therapy for NSCLC is always necessary.

Chinese populations have widely used Traditional Chinese medicines (TCM) for treatment of various diseases as a first line or complementary therapy. There are few TCM-based herbal extracts have also been reported in exhibiting potent NSCLC suppression [5,6]. TCM appears to be clinically useful, yet the underlying mechanisms remain unknown. Comprehensive understanding of the associated mechanism may aid in complementing current first-line therapy as well as fueling the discovery of new drugs. Our study subject, BL02 is an empirical formula, consists of two herbs: *Gentiana rhodantha* Franch. ex Hemsl and *Gerbera anandria*. Approximately 19 compounds are identified from *G. rhodantha*, which included several iridoids, aromatic glycosides, seco-iridoid glycosides, benzophenone glucosides, phenolic compounds, xanthone and xanthonoids [7]. Further studies reported that the leaves and flowers of *G. rhodantha* majorly consisted of loganic acid and high levels of mangiferin; whereas gentiopicroside which is high in *Gentiana* species is relatively low in *G. rhodantha* [8]. Besides, sweroside was found to be exclusively present in the root of *G. rhodantha*, suggesting the application of sweroside for characterization of different parts of *G. rhodantha* [9]. As for *G. anandria*, approximately 9 compounds were identified, which included xanthotoxin, 7-hydroxy-1(3 H)-isobenzofuranone, gerberinside, quercitin and apigenin-7-O-β-D-glucopyranoside [10].

BL02 has been commonly used to relieve the cough-like symptoms in the rural and mountainous area of West-Southern China and recently been re-discovered as a galenic formulation for hospitalized patients. Investigations to date on the use of single herbs for disease treatment are scanty. There are only a few reports postulated the use of *Gentiana rhodantha* Franch. for treatment of tuberculosis, inflammation, and cancer-like diseases [7], while *Gerbera anandria* is used to treat coughs and sore throat due to its anti-inflammatory and antibacterial effects [11,12].

Motivated by the ethnomedicinal use of BL02 by indigenous people, in this study, we investigated the therapeutic potential of BL02 on NSCLC. The *in vitro* and *in vivo* experimental studies were performed to evaluate the effect of BL02. Transcriptomics analysis was introduced to profile the gene expression of NSCLC cells treated with BL02; Network pharmacology and molecular docking analyses predicted the interaction of BL02-derived compounds and NSCLC targets. Our present findings postulated that BL02 is an alternative therapy for NSCLC, and a promising lead for the discovery of new drug specifically for NSCLC treatment. This study also demonstrated an integration model of experimental, transcriptomic and bioinformatics analyses in the identification of the novel therapeutic target of NSCLC from an adjuvant herbal formula.

2. Materials and methods

2.1. UHPLC analysis

A LC system (UHPLC, Thermo Fisher Scientific, USA) equipped with C18 column (2.1 × 100 mm) (ACE, UK) as stationary phase was used to analyze and separate the sample components of BL02. The column temperature was maintained at 30^{0}C, injection volume was 4 µl and flow rate was 1 ml/min. Mobile phase A and B were 0.05% KH_2PO_4 and 0.1% H_3PO_4 in water, and acetonitrile correspondingly. The gradient elution profile was as follows: 0–4 min, 95% A; 4–7 min, 88% A; 7–13 min, 85% A; 13–28 min, 80% A; 28–32 min, 78% A; 32–35 min, 60% A.

2.2. Orthotopic intra-thoracic NSCLC murine model

The animal procedures described in this study was approved by the Committee on the Use of Live Animals in Teaching and Research, The University of Hong Kong, Hong Kong. Two luciferase-tagged NSCLC cell lines was used to establish the mouse model [13]. The 4–5 weeks old BALB/cAnA-nude male mice were used. In brief, the 100 mg and 10 mg/kg of ketamine/xylazine- anesthetized mice were placed on the right lateral decubitus position, and the left chest wall was exposed by incising the skin on top of the mid-axillary line. The mixture of NSCLC cells suspended in PBS and Matrigel matrix (1:1) was injected through the lateral dorsal axillary line with approximately 6 mm in depth into the left thorax. After tumor cell inoculation, the skin was closed and the mice were turned to the left lateral decubitus position. After one week of post-surgery, the mice were subjected to IVIS spectrum imager (Perkin Elmer Inc., USA) for bioluminescence signal, as indicative of successful model establishment. After that, the mice showing positive signal were randomized into four groups (n = 6): control vehicle group, erlotinib group (25 mg/kg, p.o.), BL02 low dose group (BL02-L, 0.5 g/kg, p.o.) and BL02 high dose group (BL02-M, 1 g/kg, p.o.). Tumor growth was monitored throughout the intervention period using IVIS spectrum imaging system.

2.3. Cell culture

The human NSCLC cell lines HCC827 and A549 tagged with luciferase reporter gene were both purchased from Japanese Collection of Research Bioresources cell bank. HCC827 was cultured in RPMI 1640 medium (Gibco, USA) while A549 was cultured in MEM medium and both culture media were supplemented with 10% of fetal bovine serum and 1% of penicillin/streptomycin. The cells were kept in 37^{0}C humidified incubator equipped with 5% CO_2.

2.4. Transwell invasion assay

For the migration assay, the BL02 pre-treated NSCLC cell lines at a density of 1×10^5 were seeded on the upper chamber of transwell (8 µm pore size, Corning, USA) coated with Matrigel matrix (5 mg/ml, 1:2 diluted with cold PBS, BD Bioscience, USA). The lower chamber was filled with 750 µl of medium supplemented with 10% fetal bovine serum. After 48 h of incubation, the cells in the upper chamber were wiped with a cotton swab. The basolateral cells that invaded through the chamber were further fixed and stained with crystal violet for 2 h. The number of migrating cells was then quantified as the average of five fields of view per chamber under an inverted microscope.

2.5. Wound healing assay

NSCLC cells were seeded on the multi-wells plate and allowed to grow till 100% confluence. A 10 µl pipette tip was then used to make a vertical wound across the monolayer of cells. Then, the cells were co-incubated with or without BL02 for 24 and 48 h. By the end of the experiment, the cells were visualized under an inverted microscope for any wound closure.

2.6. RNA isolation and transcriptomic analysis

The RNA of NSCLC cells treated with BL02 or vehicle was isolated using TRIzol reagent (Takara, Japan). The RNA quality and purity were determined by spectrophotometer (Nanodrop) and agarose gel electrophoresis. After cDNA library construction, the cDNA quality and integrity were determined by Agilent 2100 Bio-analyzer (Agilent, USA). The cDNA was then sequenced by Illumina HiSeqTM2500/Miseq TM system (Illumina, USA). The raw data from sequencing was transformed into raw reads using Illumina CASAVA.

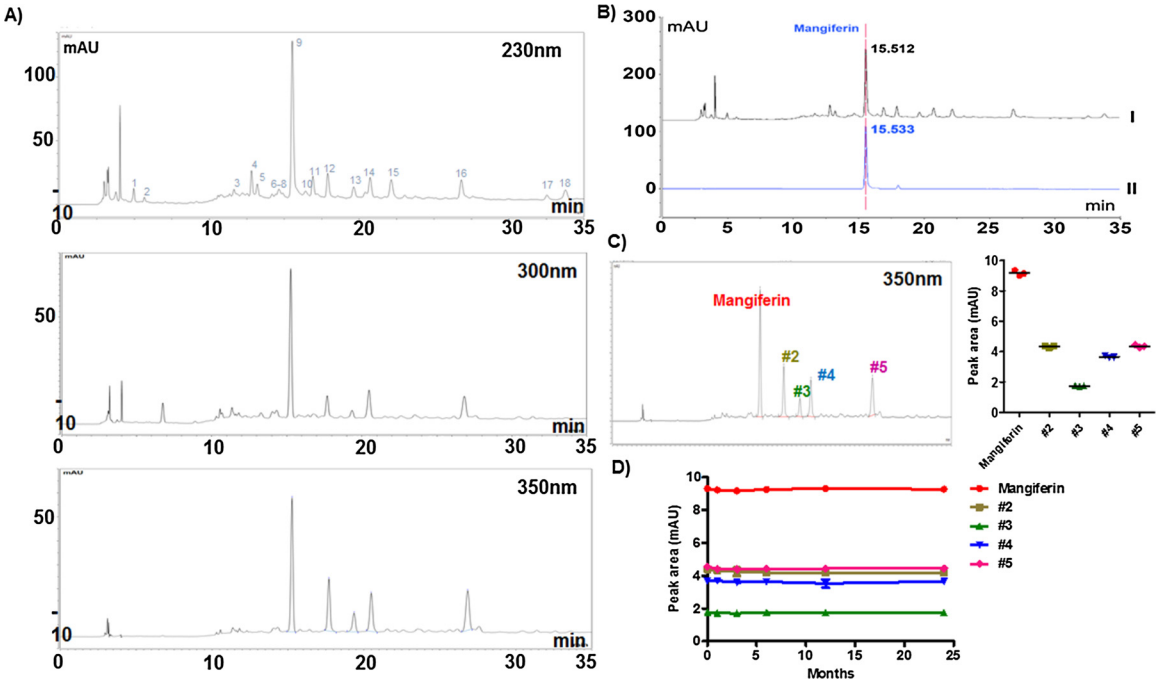

Fig. 1. The chemical fingerprinting of BL02. (A) The UV chromatogram of BL02 at different detection wavelengths. (B) The chromatogram at 230 nm of (I) BL02 and (II) mangiferin with labeled peak retention time. (C) Chemical profile of BL02 at 350 nm with 5 major chromatographic peaks in common. The area of the 5 peaks was consistent within batches of production (n = 3). (D) The area of the 5 chromatographic peaks was consistent throughout 24 months of storage.

2.7. Identification of chemical components, prediction of interaction between compounds and targets and network construction

To identify the active constituents of *Gentiana rhodantha* and *Gerbra anandria*, databases including BATMAN (a Bioinformatics Analysis Tool for Molecular mechANism of TCM) [14], TCMSP (TCM Systems Pharmacology Database and Analysis Platform) [15], TCM-ID (TCM-Information Database) [16–18], TCM@Taiwan (TCM Database@Taiwan) [19], HIT (Herbal Ingredients' Targets Database Introduction) [20] as well as associated literatures were thoroughly searched. The compounds associated oral bio-availability (OB), Caco-2 permeability as well as drug-likeness (DL) were extracted from TCMSP database. All these data were summarized in Supplementary table 1. Potential molecular targets of each identified chemicals were then identified through various databases, which include BATMAN-TCM, STITCH [21] and Drugbank [22]. The literature review was also done to complement the findings from the above databases. The list of putative targets of identified chemicals in BL02 was listed in Supplementary Table 2. On the other hand, the list of genes that altered in NSCLC was extracted from the database Genecards [23]. Then, the NSCLC-regulated genes were further merged with the BL02-regulated putative targets; the overlapping genes as indicative of BL02-regulated NSCLC genes were sorted out and summarized in Supplementary Table 3. All the target proteins by BL02 were then subjected to DAVID bioinformatics resources [24] for functional enrichment analysis. The network bridging the active ingredients of BL02 and NSCLC-associated targets (Supplementary Fig. 1) and ClueGo network that identified strongly correlated pathway regulated by BL02 was established by Cytoscape 3.7.0 software.

2.8. Immunofluorescence staining

The NSCLC cells pre-treated with BL02 or vehicle was fixed with 4% of paraformaldehyde and permeabilized with 0.1% of Triton X-100 in PBS. The cells were then incubated with Phalloidin-ifluor 488 (Abcam, USA) for 30 min. It was followed by counterstain with DAPI before slide mounting with fluorescent mounting medium (Dako, Denmark). The phalloidin-stained specimens were visualized under a confocal microscope (Carl Zeiss LSM 780, Germany).

2.9. Immunoblotting

The pre-treated cells were lysed in RIPA buffer supplemented with 1% of PMSF. Protein concentration was determined by the Bio-rad protein assay dye (Bio-rad, USA). An equal amount of protein was loaded on SDS-PAGE and electrotransferred to PVDF membrane. The membrane was then blocked with 5% bovine serum albumin containing buffer for 2 h, followed by incubation with anti-cdc42 (#2462), anti-Rap1A/Rap1B (#4938) and anti-GAPDH (#5174) (Cell Signaling Technology, USA) overnight. On the next day, HRP-conjugated secondary antibody was applied to the membrane for 2 h before band visualization with ECL detection kit (Sigma, USA) under a chemiluminescence system (Bio-rad, USA).

2.10. Active Rap1 detection assay

The active Rap1 detection kit was used according to the manufacturer's protocol (#8818, Cell Signaling Technology, USA). Briefly, 50% of resin slurry was added to the spin cup with a collection tube. After that, 20 µg of GST-RalGDS-RBD protein was added to the spin cup containing glutathione resin, followed by 500 µg of cell lysates and the mixture was incubated in the spin cup at 4°C. After an hour of co-incubation, the spin cup was centrifuged and washed for three times with the indicated buffer. Lastly, to elute the samples, 2X SDS sample buffer was applied to the resin. The eluted samples were then subjected to immunoblotting analysis.

2.11. Statistical analysis

All data was present as mean ± SD. Data between two groups was analyzed by Student's *t-test*, while one-way ANOVA was used to analyze data for more than two groups. A *p*-value lower than 0.05 was considered as statistically significant.

3. Results

3.1. Quality assessment of BL02

The herbal formulation, BL02 is composed of two herbs, the *Gentiana rhodantha* Franch. ex Hemsl and *Gerbera anandria*. There have been some, but limited literature reported on the chemical composition of the two herbs. Here we established the chemical profile of BL02 with high-performance liquid chromatography. There were approximately 18 representative peaks observed on the chromatogram of BL02 (Fig. 1a). By comparing the retention time and UV spectrum, we identified that mangiferin as the main compound of BL02, which was associated with the highest chromatographic peak (Fig. 1b). In correlation with the respective peak area, the total content of mangiferin in BL02 was about 1.7%, which indicated approximately 100μM of mangiferin in 2500 mg/ml of BL02. By configuring chromatograms from different batches of BL02, we established the chemical profile of the herbal formula with 5 major chromatographic peaks in common, one of which referring to mangiferin (Fig. 1c). Quality consistency was measured by calibrating the area of five common peaks in the chromatograms of 3 different batches of BL02, and the result showed that chemical composition of BL02 was consistent within batches of production (Fig. 1c). To validate the stability of the herbal formulation, a similar batch of formulation was subjected to chromatography analysis at different time-point (0, 1, 3, 6, 12, and 24 months). Quality of BL02 was relatively stable up to 24 months of storage (Fig. 1d).

3.2. BL02 suppresses in vivo growth of non-small cell lung carcinoma

Although BL02 has been empirically used for the treatment of lung carcinoma, however, the up-to-date scientific evidence on the tumor inhibitory effect of BL02 remains scanty. To systematically study the role of BL02 in non-small cell lung cancer (NSCLC) growth, we introduced an orthotopic NSCLC murine model by intra-thoracically injecting the lung cancer cell lines to the mice, accompanied with oral administration of different concentrations of BL02 on alternate days for seven weeks. As NSCLC is classified based on its *EGFR* mutation in the clinical setting to triage patients towards appropriate treatment, we examined the effect of BL02 on NSCLC animal models with and without *EGFR* mutation. Two cancer cell lines which one of them is *EGFR*-wild type, A549 and another one is *EGFR*-mutant, HCC827 cells were used (Fig. 2a). *EGFR*-targeted therapy erlotinib was used as a positive control. The *in vivo* growth of tumor derived from luciferase-tagged NSCLC cell lines was monitored by an alternate week of *in vivo* imaging. In NSCLC tumor established from *EGFR*-wild type A549 cells, erlotinib treatment had minimal effect on the lung tumor progression. Starting from the week three after tumor implantation, treatment of BL02 at doses of 0.5 g/kg (BL02-L) and 1.0 g/kg (BL02-M) every day showed significant A549-derived tumor regression and retarted tumor growth throughout the intervention period (Fig. 2b). However, overall survival was not enhanced in the mice treated with 1.0 g/kg of BL02 compared to erlotinib treatment, whereas both the mice treated with erlotinib and 0.5 g/kg of BL02 had better survival relative to the control mice (Fig. 2c). The body weight of mice was increased continuously throughout intervention (Fig. 2d). By the end of the experiment, the tumor-bearing mice were sacrificed, and the luciferase emitted signal in lung tissue indicative of tumor size was measured. Notably, mice administrated with BL02 showed significantly smaller tumor size relative to mice of control group (Fig. 2e). A similar observation in tumor suppression and redundant tumor progression were obtained after 3-weeks of BL02 treatment in mice with *EGFR*-mutant HCC827-derived tumor (Fig. 2b and e). It was observed that the HCC827-derived tumor growth was completely abolished after two-weeks of erlotinib intervention. Similarly, the body weight of tumor-bearing mice substantially grew throughout the intervention period (Fig. 2d). In sum, up to 1 g/kg of BL02 is safe and effective to ameliorate NSCLC tumor growth *in vivo*.

3.3. BL02 inhibits lung cancer cell invasion and migration in vitro

To extend our understanding, we examined if the *in vivo* inhibition of NSCLC tumor progression by BL02 was associated with its direct cytotoxicity to NSCLC cells. The MTT assay postulated that up to 5 mg/ml of BL02 was not lethal to the A549 cells, while incubation of BL02 at a dose up to 3 mg/ml had minimal effect to the viability of HCC827 cells (Fig. 3a). These data suggest that A549, the *EGFR*-wild type cells may be more resistant to BL02 treatment compared to *EGFR*-mutant HCC827 cells. Since the progression of NSCLC may also be related to the invasion of tumor cells within lung tissue, we further explored if BL02 affected the cancer cell motility. Intriguingly, at doses (1.25 mg/ml and 2.5 mg/ml) far lower than its cytotoxic dose, BL02 significantly blocked the chemotactic ability of both A549 and HCC827 cells (Fig. 3b). Consistently, the wound healing assay also revealed that BL02 treatment decreased lung cancer cells migration at a dose-dependent manner (Fig. 3c). Histological observation in both animal models revealed that BL02-treated mice showed smaller lung lesions compared to control mice (Fig. 3d). The alveolar structures of control mice were highly invaded by cancer cells and displayed high vascularity. On the other hand, observations of encapsulated and well-defined margin in BL02-treated lung tumor revealed the inhibition of tumor cell invasion into healthy lung tissue by BL02 regardless of the *EGFR* status. All these suggest that the *in vivo* tumor regression by BL02 might be more likely related to its inhibition on cell migration and invasion, rather than direct cytotoxicity. Overall, the non-toxic dose of BL02 inhibited NSCLC chemotactic and migratory ability.

3.4. Transcriptomic analysis identified the involvement of cellular adhesion-related signaling in the inhibitory effect of BL02 on NSCLC

To further understand the mechanism underlying the inhibitory effect of BL02 on NSCLC growth, both cell lines A549 and HCC827 with or without BL02 treatment were subjected to transcriptomic profiling analysis. There were in total 13,788 genes and 13,027 genes (FKPM more than 1) detected in non-treated A549 and HCC827 cells, respectively. It was noted that 471 differentially expressed genes between non-treated and BL02 treated groups in A549 cells, while 425 genes were differentially expressed in BL02 *versus* the control group in HCC827 cells (Fig. 4a). Observing the potent inhibition on NSCLC progression by BL02 in both cells, we further sorted out the overlapping genes that were regulated in both A549 and HCC827 cells following BL02 treatment. Intriguingly, only 43 common genes in both lung cancer cells were regulated in the presence of BL02. BL02 up-regulated six of the genes (Fig. 4b). Gene ontology analysis revealed that BL02 treatment was correlated to a significant enrichment of genes involved in biological processes such as cell communication and signal transduction. These genes mainly participated in the molecular functions of protein binding and growth factor activity (Fig. 4c). Further pathway crosstalk analysis postulated that BL02 majorly regulated the genes involved in EGFR signaling (ErbB receptor, EGFR-dependent pathway) and cell adhesion pathway (Cdc42 signaling and beta3 integrin cell interaction) (Fig. 4d). NSCLC is the malignancy often harboring of *EGFR* mutation, and thus current tyrosine kinase inhibitors targeting at EGFR such as gefitinib and erlotinib are effective in specific subsets of NSCLC [25]. All these data postulated that BL02 might inhibit NSCLC growth through mediating EGFR and cellular adhesion-related pathways.

3.5. Network pharmacology predicted the possible compound-target interaction in the inhibitory effect of BL02 on NSCLC

To date, computational based network pharmacological approach has been extensively used for dissection of the pharmacological actions of the multi-components TCM formulas on complicated disease such as cancer. This approach also identify the potential synergistic actions or multi-levels interactions of multiple components on the disease

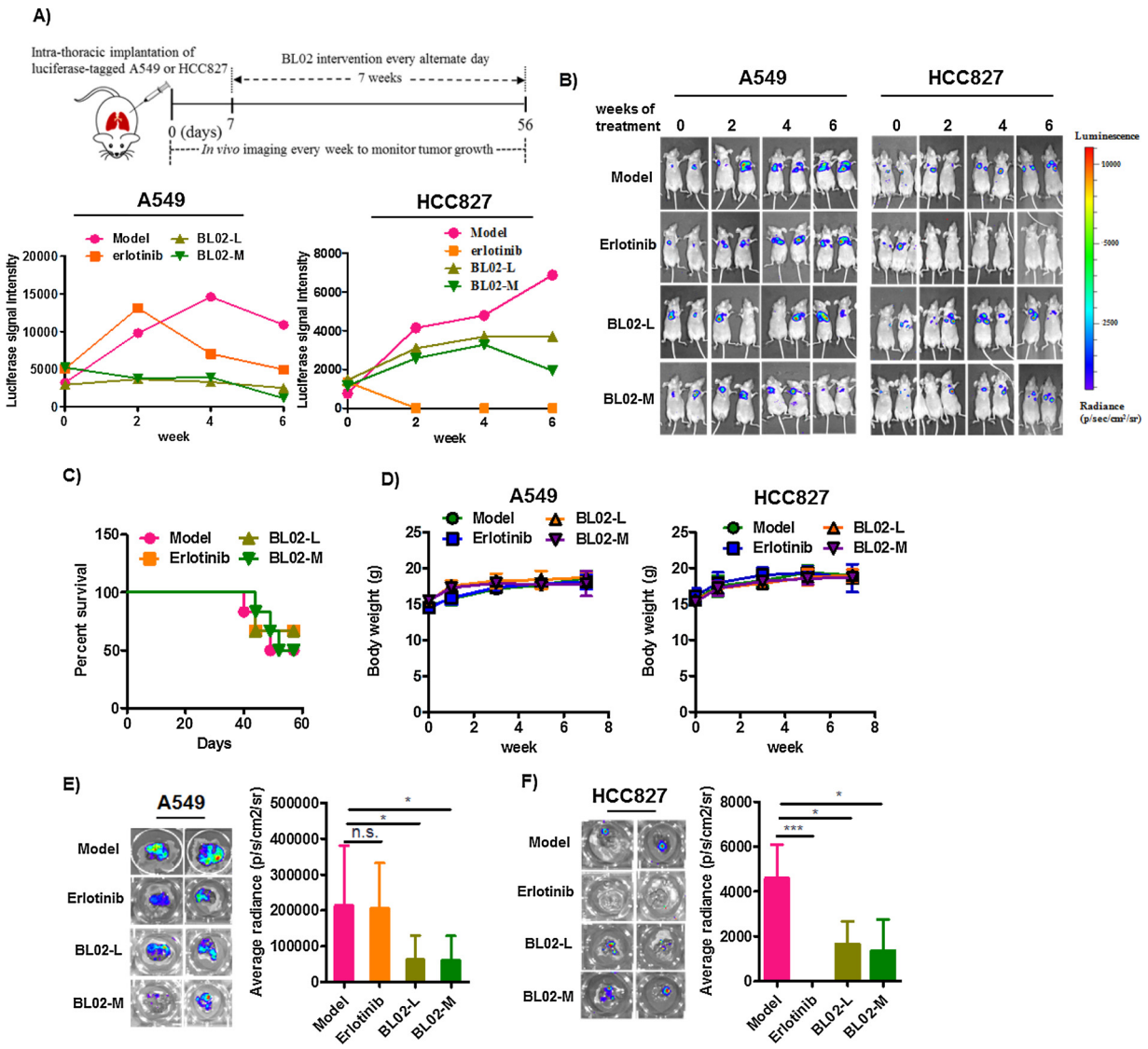

Fig. 2. BL02 suppresses *in vivo* growth of NSCLC. (A) The schematic flowchart of the orthotopic animal study. (B) Representative figures of orthotopic NSCLC-implanted mice in different treatment groups. Biweekly monitoring of tumor growth was performed by subjecting the tumor-bearing mice to IVIS spectrum imager. Reduction in the intensity of luciferase signal were observed following 4-weeks of BL02 intervention. (C) Kaplan-Meier survival curve of tumor-bearing mice with or without treatment ($n = 6$ per group). Mice treated with erlotinib and low dose BL02 showed better survival than control group of mice. (D) The body weight of mice throughout the course of intervention. (E) Representative bioluminescence images indicative of A549 cells derived lung tumor harvested after 8 weeks of growth. Mice intervened by BL02 showed significant smaller tumor size relative to control mice. (F) Representative bioluminescence images of HCC827 cells derived lung tumor. Erlotinib treatment completely abolished HCC827 derived tumor growth, and BL02-treated mice showed significant smaller tumor size compared to control mice. $^{*}p < 0.05$, $^{***}p < 0.001$.

treatment or prevention [26]. Therefore, observing the limited compounds was identified from the chromatogram of BL02, we have included the network pharmacological investigation to determine the chemical ingredients in BL02 as well as the potential active ingredients that involved in the NSCLC suppression. Firstly, to identify the constituents of the *Gentiana rhodantha* and *Gerbra anandria,* the online database, as well as its associated literatures, were thoroughly searched, and all identified chemical compounds from extracts of these two herbs were integrated and summarized in Supplementary Table 1. There are in total 74 compounds identified in BL02, in which 56 compounds from *Gentiana rhodantha* and 20 compounds from *Gerbra anandria.* It was also noted that quercitin and β-sitosterol are co-existed in both herbs. Since not all compounds possess pharmaceutical property, we further analyzed the oral-bioavailability, Caco-2 permeability, and drug-likeness of the constituents. It was observed that most of the compounds from the two herbs have low oral-bioavailability (lower than 30%) and Caco-2 permeability (lower than 0.4 cm/s) as indicative of compound absorption level (Fig. 5a). Surprisingly, most of the compounds showed high drug-likeness (more than 0.1%), suggestive of the higher possibility of the "drug-like" property of the compounds in terms of solubility and chemical stability [27].

We next identified 966 molecular targets corresponding to the compounds from BL02 (Supplementary Table 2). On the other hand, there are 3015 genes extracted from Genecard, which are dysregulated in NSCLC, and 363 of them are overlapping with the molecular targets of BL02 (Fig. 5b). These results suggested that BL02 treatment may be very potent against NSCLC. Further gene ontology analysis postulated that these genes are enriched of the pathway related to cancer invasion and growth such as TRAIL signaling, sphingosine-1-phosphate pathway, integrin-linked kinase signaling, and cdc42 signaling (Fig. 5c). Through constructing the network between BL02-regulated molecular targets and NSCLC-associated molecules (Supplementary Fig. 1), it was observed that 57 NSCLC-related genes were targeted by two or more compounds of BL02 (Fig. 5d & e). Besides, KEGG-dependent ClueGO

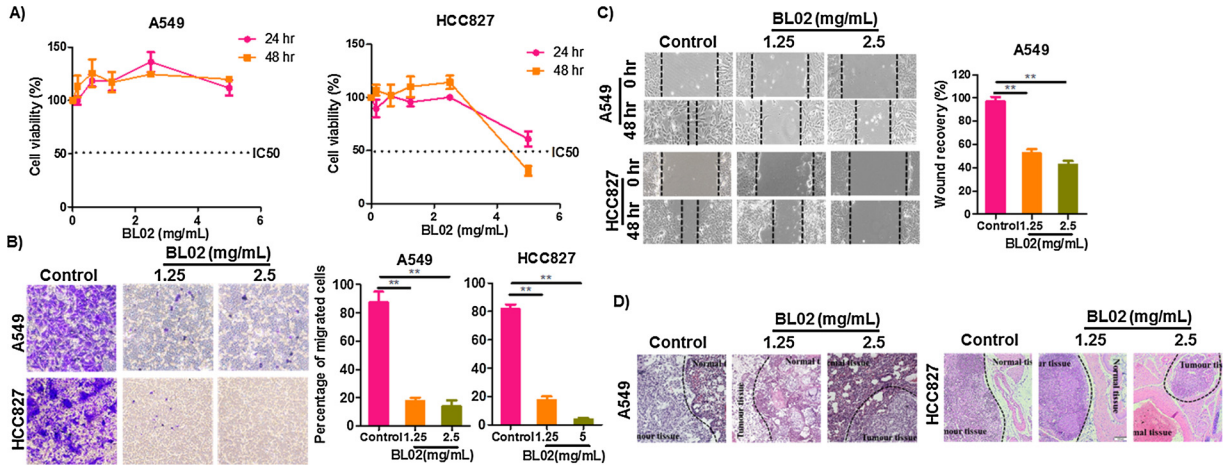

Fig. 3. BL02 inhibits lung cancer cell invasion and migration *in vitro*. (A) The cytotoxicity curve of A549 and HCC827 cells following BL02 treatment in time- and dose-manner. BL02 at doses up to 4 mg/ml induced no significant *in vitro* toxicity on NSCLC. (B) BL02 at non-toxic doses significantly suppressed the migration of NSCLC cells. (C) 48 h-incubation of BL02 significantly inhibited the gap closure of NSCLC cells at dose-dependent manner. (D) H&E staining of tumor-bearing lung sections with or without BL02 treatment. The invasive edge between tumor and normal lung tissue was illustrated by the black dashed line. It was observed that the lung alveoli of control mice were highly invaded by cancer cells; whereas BL02-treated mice showed smaller tumor mass in lung. $^{**}p < 0.01$.

analysis revealed that most of these shared genes regulated the cancer cells migration and cell-cell communication (Fig. 5f). These findings were consistent with our experimental *in vitro* and *in vivo* data that BL02 inhibited NSCLC primarily through retarding the cancer cell motility. In sum, the bioinformatics data showed that the suppression of NSCLC by BL02 might involve its regulation on cancer migration and growth-related signaling pathways.

3.6. Rap1/cdc42 signaling pathway is responsible for the BL02 mediated NSCLC suppression

Pathway analysis from both transcriptomic and bioinformatics analysis postulated that cdc42 signaling might be the responsible pathway mediating NSCLC inhibition by BL02. Cdc42 is one of the small GTP-binding proteins of the Rho GTPase family [28] and

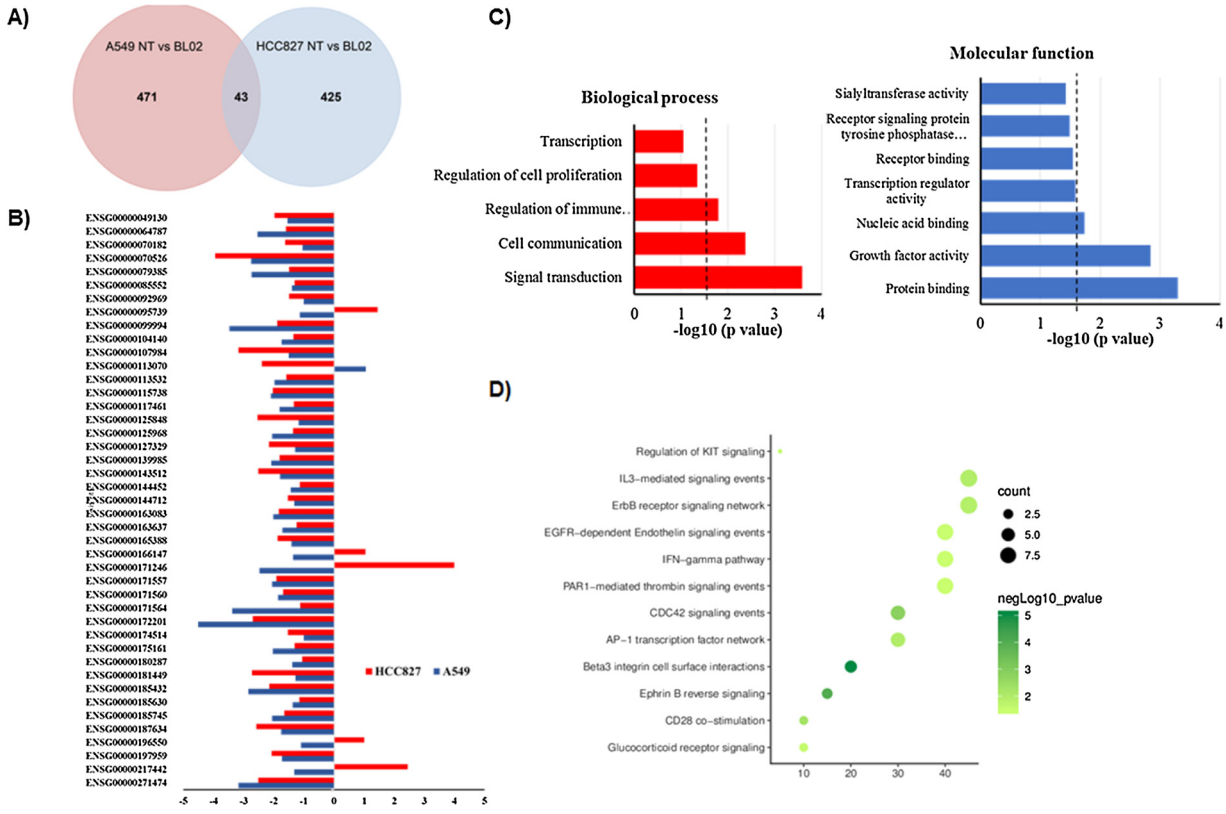

Fig. 4. Transcriptomic analysis identified the involvement of cellular adhesion-related signalling in BL02-mediated NSCLC inhibition. (A) Venn analysis on differentially expressed genes between non-treated and BL02-treated A549 and HCC827 cells. There was 43 genes co-regulated by BL02 in both NSCLCs. (B) The BL02 co-regulated genes profile in NSCLCs. 37 of the genes was down-regulated by BL02, while 6 of them was up-regulated. (C) Gene ontology analysis on the involvement of major biological process and molecular function of common genes regulated by BL02 on NSCLCs. (D) KEGG pathway crosstalk analysis showed that BL02 majorly regulated the genes involved in EGFR signalling (ErbB receptor, EGFR-dependent pathway) and cell adhesion pathway (CDC42 signalling and beta3 integrin).

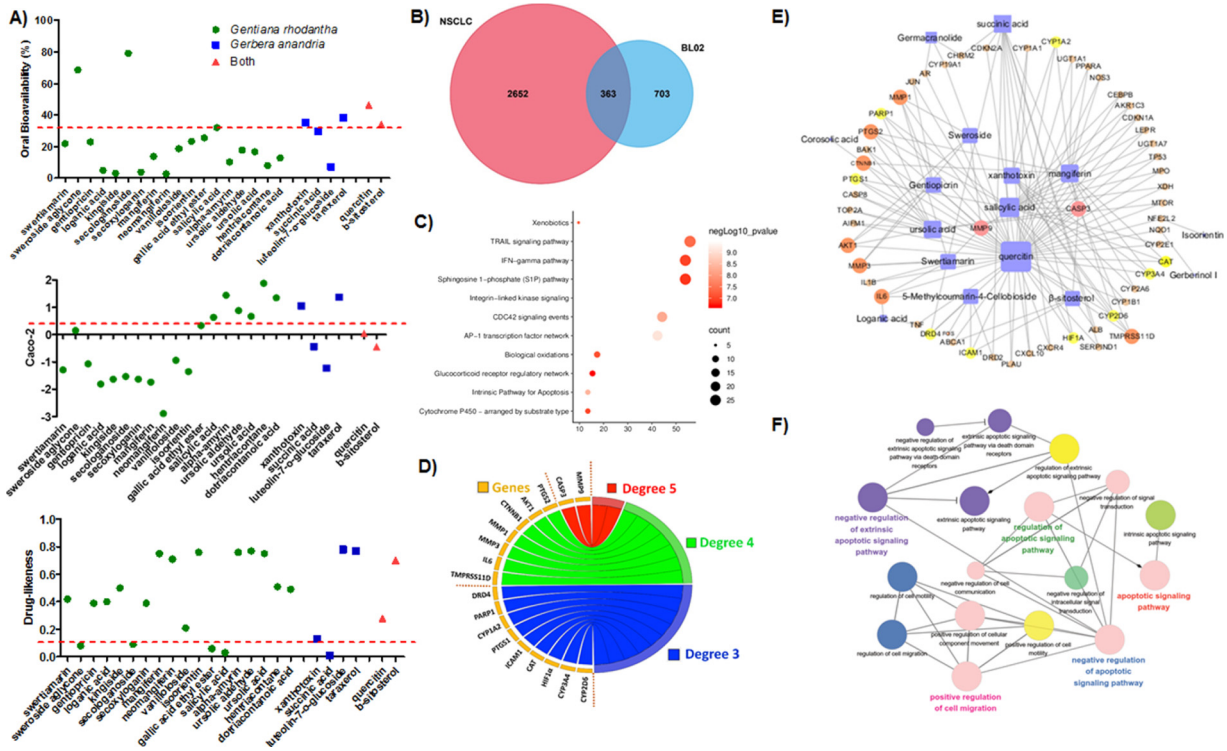

Fig. 5. Network pharmacology predicted the possible compound-target interaction in BL02 mediated anti-NSCLC. (A) The oral-bioavailability, Caco-2 permeability and drug-likeness of the identified constituents from BL02. Oral-bioavailability < 30%, Caco-2 permeability < 0.4 cm/s or drug-likeness > 0.1% suggestive of the "drug-like" property of the active molecules as therapeutic agents. Quercitin and β-sitosterol are the compounds co-existed in *G. rhodantha* and *G. anandria*. (B) Venn analysis on NSCLC-regulated genes and putative molecular targets of BL02. There were 363 NSCLC-targeted genes regulated by BL02. (C) KEGG pathway crosstalk analysis showed that BL02-regulated NSCLC genes are enriched of the pathway related to cancer invasion and growth. (D) Circos plot illustrates the genes regulated by more than 3 compounds of BL02. Degree of a node indicates the number of compounds are connected to the specific gene. (E) The compound-target network illustrates the interaction between active components of BL02 and NSCLC-targeted genes. The blue square box indicates active compounds of BL02, while the orange and yellow circles indicate NSCLC genes. The size of the box or circle postulate the number of genes or compounds connected to it. (F) The network reveals most of the common genes regulated by BL02 involves cancer cells migration and cell-cell communication. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

facilitates the actin filament assembly and reorganization in cancer cells. We thus explored if BL02 affect the actin filament polymerization in lung cancer cells. It was observed that the presence of BL02 actively disrupted the phalloidin-labeled actin cytoskeleton organization in HCC827 and A549 cells (Fig. 6a). Further immunoblotting analysis confirmed that BL02 intervention decreased cdc42 protein expression. Besides, Rap1 GTPases, another member of the GTP-binding protein of Ras superfamily, which acts as the upstream molecule of cdc42 signaling, also controls the actin organization [29]. Indeed, the Rap1 protein expression was reduced in BL02 pre-treated lung cancer cells (Fig. 6b). Further pull-down assay using GST-RalGDS-RBD fusion protein confirmed that activated GTP-bound Rap1 was decreased following BL02 intervention. The p21-activated kinases (PAKs) has been long recognized as the effectors of Cdc42/Rap1. Binding of the GTP-bound form of Rap1 to the kinase domain of PAK activated group I PAK activity [30]. Further immunoblotting result also showed that BL02 significantly suppressed PAK1 expression (Fig. 6b). After confirming the role of BL02 in mediating NSCLC suppression through Rap1/cdc42/PAK1 inhibition, we further assessed the interactive activities of identified BL02 constituents with Rap1 by *in silico* molecular docking analysis. Among all, 5-methylcoumarin-4-cellobioside and mangiferin showed most potent binding potency with Rap1a protein (△G:-8.41 and -8.39 kcal/mol) (Fig. 6c & Supplementary Table 4), indicating that both of them may be the responsible molecules for the anti-NSCLC effect of BL02 *via* Rap1 suppression. Overall, our findings unveiled that inhibition of Rap1/cdc42 signaling by BL02 suppressed NSCLC migration and growth.

4. Discussion

Molecularly targeted therapy such as gefitinib and erlotinib is the leading and effective treatment for NSCLC patients harboring of *EGFR* mutation [31]. However, NSCLC cells harboring a mutation in *EGFR* are not commonly found in patients from Europe and America, and plenty of clinical trials have even revealed on failure in treatment or even worsening condition of NSCLC patients receiving tyrosine kinases inhibitors (TKIs) [32,33]. For patients unresponsive or resistant to TKIs, treatments only gain marginal health benefit, and the disease still progresses rapidly [34]. Discoveries of new agents, especially for these group of patients, is emerging. Numerous empirical records in traditional medicine have brought out the ancient wisdom in the use of medicinal herbs, which contain a series of the compound that are promising to become leads in drug discovery [35]. BL02, as an empirical formula has been commonly used to relieve the NSCLC-like symptoms in the rural and mountainous area of West-Southern China and recently been re-discovered as a galenic formulation for hospitalized patients. In this study, we managed to elaborate on the scientific evidence of the anti-tumor effect of BL02 on human NSCLC. Combining transcriptomic analysis and system biology, we identified that several compounds in BL02 could target the Rap1 protein of NSCLC cells and thereby inhibit NSCLC progression (Fig. 6d). Notably, the primary identified compound mangiferin from *G. rhodantha*, which yields the highest abundance in BL02, exhibited high affinity to Rap1. Whereas 5-methylcoumarin-4-cellobioside from *G. anandria* also showed potent binding with Rap1 to inhibit its expression and activity. Our and other previous studies have postulated the potent anti-tumor effect of

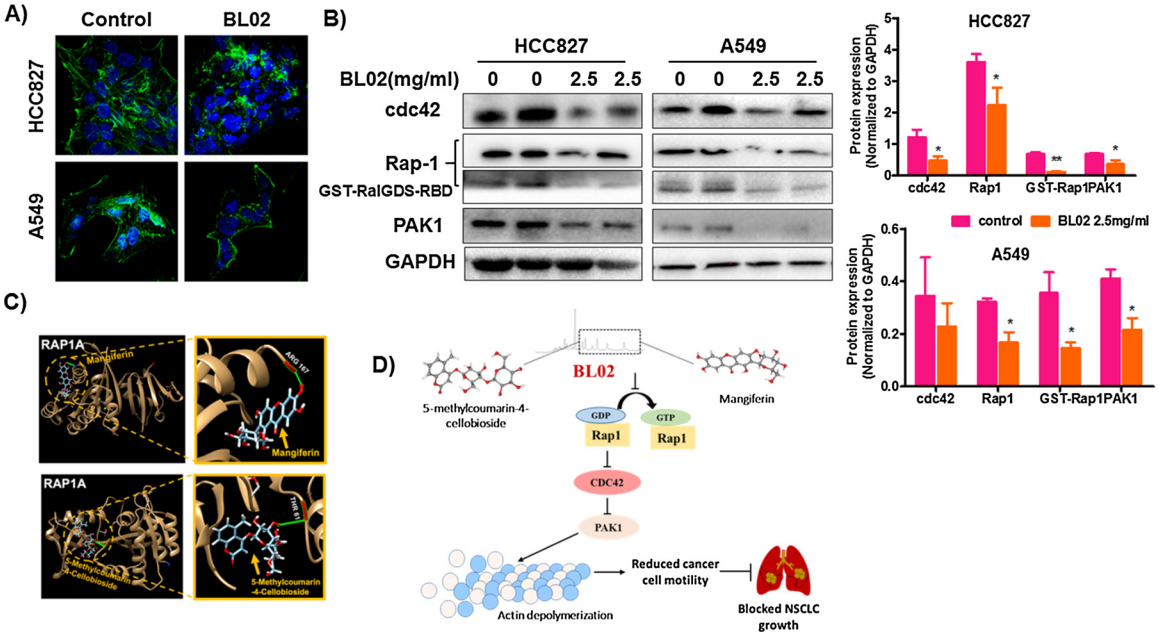

Fig. 6. Rap1/cdc42 signaling pathway is responsible for the BL02-mediated NSCLC suppression. (A) Microscopy images of cultured NSCLC cells with or without BL02 treatment. The cells were stained with Phalloidin-ifluor 488 (green) and DAPI (blue). BL02 actively disrupted the phalloidin-labelled actin cytoskeleton organization in NSCLCs. (B) Expressions of cdc42, Rap-1, PAK1 and GAPDH in HCC827 and A549 cells with or without BL02 intervention. GST-RalGDS fusion protein was used to determine the level of GTP-bound active Rap1 in NSCLCs with or without BL02. Quantification of band intensity is shown on the right bar chart. (C) The structure of interaction between Rap1a protein and mangiferin or 5-methylcoumarin-4-cellobioside. Right panel shows the magnified images of potential binding clusters of Rap1a by the compounds. (D) Schematic diagram postulated the inhibitory effect of NSCLC by BL02 through deactivating Rap1/cdc42/PAK1 signaling. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

mangiferin [36,37], but there were no studies-to-date showing the interaction between mangiferin and Rap1 protein. As for 5-methylcoumarin-4-celloboside, its associated pharmacological effect has not yet been reported, despite bio-informatics database postulated the association of 5-methylcoumarin-4-celloboside with a high abundance of putative molecular targets. Although our study could not experimentally validate the effect of all compounds due to their low availability, the computation prediction still provides significant clues on the potential of 5-methylcoumarin-4-celloboside and mangiferin as the novel therapeutic agents for NSCLC with various molecular subtypes.

In our studies, we observed that the expression and activity of Rap1 in NSCLC cells could be suppressed by BL02 treatment. Previous studies have elaborated the role of Rap1 in NSCLC progression and resistance to treatment. Working as a GTPase switch, the constitutively activated Rap1 could promote the NSCLC cell spreading through up-regulating Erzin [38]. Hypoxia in the tumor microenvironment seemed to be the promoting factor of Rap1 activity, which then activated the cell migration in NSCLC [39]. Modulating Rap1 expression could increase the sensitivity of NSCLC cells to chemotherapeutic agents [40], which is associated with decreased Akt activity and induced HDAC8 expression [41]. The Rap1-mediated resistance of NSCLC cells was related to activation of NF-κB and anti-apoptotic Bcl-2 protein expression [42]. Although Rap1 activity requires its activation through GTPase switch, we found that Rap1 expression was significantly increased in NSCLC, and BL02 co-incubation suppressed it. Independent to its role as a GTPase, Rap1 expression was also found involved in various biological activities. Notably, Rap1 protein could physically and directly associate with embryonic stem cell marker, Zinc finger, and SCAN domain-containing 4 genes (Zscan4), resulting in increased telomere length and cancer cell immortality [43]. Yang et al. suggested a direct interaction between Rap1 and NF-κB protein, in which the GTPase switch of Rap1 might not be necessary for the interaction [44]. Although most of the current studies do not exclusively identify the distinct functions of Rap1 protein independent to its GTPase activity, it is no doubt that suppressing its expression at the same time magnifies the inhibition of Rap1-activated oncogenic pathways and cancer progression. As postulated from our in silico molecular docking analysis, the two compounds, 5-methylcoumarin-4-cellobioside and mangiferin from BL02 showed potent binding to the Rap1 pocket at the residues of Thr61 and Arg167 correspondingly. Further reduction of Rap1 GTPase activity as observed from immunoblotting assay suggested that the potential competition between 5-methylcoumarin-4-cellobioside and mangiferin with GTP. This phenomenon was supported by a previous study showing that the binding of a small molecule to Cdc42 pocket led to Cdc42 deactivation and its-regulated filopodia inhibition [45]. The direct binding of BL02 on the Rap1 protein led to the downstream effector deactivation and as a result of actin filament disruption.

Apart from NSCLC, many other cancers were also constitutively expressing high levels of Rap1. The Rap1 was found to only express in colorectal cancer tissue of patients rather than matched adjacent normal tissues [46], and high expression of Rap1 was associated with poor overall survival and prognosis of patients. The same was also observed in tumor tissues of oral cavity squamous cell carcinoma [47] and ovarian cancer [48] patients, suggesting the correlation of Rap1 expression with clinical significance. Besides, few recent studies have postulated the involvement of miRNAs in negatively regulating the transcript of Rap1, which confirmed the tumor-promoting function of Rap1 [49,50]. All these evidence strongly support the role of Rap1 as a potent oncoprotein for cancer growth and progression; and the potential targeted candidate for tumor suppression.

Our study noticed that BL02 could suppress Rap1 in NSCLC cells with or without *EGFR* mutation. EGFR signaling was reported to affect the Rap1 activity. Treatment of EGF could stimulate the conformation change of Rap1 protein through the dissociation of the CrkII-C3G complex [51]. Overexpression of EGFR, therefore stimulated the conformation change of Rap1 [52]. Further analysis showed that activation of EGFR could induce Src-dependent phosphorylation of p130CAS, which assembled CAS/Nck1 complex to promote Rap1 signaling [53]. These studies suggested that Rap 1 activity may be required as a downstream effector for the EGFR signaling as an oncogenic pathway

regardless of the mutation status. Indeed, activation of Rap1 conferred the resistance of NSCLC cells to EGFR inhibitor gefitinib [54]. Besides, Rap1 signaling has been implicated in the sensitivity of cancer cells towards chemotherapeutic drugs [40], and later studies proved that Rap1 activated as a result of gene alterations in cancer cells. For instance, activating mutation in ALK receptors led to enhanced Rap1 activity in neuroblastoma [55]. It was also observed that the constitutive expression of Rap1 following CRKL overexpression might be another secondary resistance pathway of NSCLC towards EGFR inhibitor. [54]. Moreover, Keller et al [56] showed that Rap1 regulation highly dependent on the KRAS mutation status, which indicated the association between Rap1 activity and sensitivity of cancer cells to targeted therapy. Our current study postulated the inhibitory effect of BL02 on Rap1 at both expression and activity level, and this may suggest the potential of BL02 as an adjuvant treatment in improving the sensitivity of EGFR inhibitor in NSCLC. More investigations are needed to warrant the conclusion of whether cdc42/Rap1 inhibition by BL02 may be a potential therapeutic target against resistant NSCLC.

5. Conclusion

As a conclusion, in this study, we reported that BL02, an empirical formulation composing of two herbs, *Gentiana rhodantha* Franch. ex Hemsl and *Gerbera anandria* were relatively stable in different batches of production as well as storage up to 24 months. Further *in vivo* experimental study confirmed that BL02 at a dose up to 1 g/kg was safe and effective to inhibit NSCLC tumor growth regardless of the tumor molecular subtype. Besides, at non-toxic treatment, BL02 was able to inhibit NSCLC migration and invasion, suggesting that BL02 suppressed NSCLC through restricting cancer cell motility. The transcriptomic analysis further showed that inhibition of NSCLC by BL02 involved EGFR and cellular adhesion-related pathways. In addition, bioinformatics analysis predicted the possible interactions of the compound of BL02 and NSCLC-mediated molecular targets and validated that the regulation on cancer migration and growth-related signaling pathways involves in BL02-mediated anti-NSCLC. The deactivation of Rap1/cdc42 signaling by BL02 depolymerized the actin cytoskeleton in NSCLC, thereby restricted NSCLC motility. These effects may be primarily mediated by the direct binding of BL02-containing 5-methylcoumarin-4-cellobioside and mangiferin to Rap1 protein. Taken together, our study postulates that BL02 as a promising alternative therapy for NSCLC patients, primarily through suppressing Rap1/cdc42 signaling regulated actin polymerization.

Declaration of Competing Interest

YF received research grants of BL02 from Bailing Pharmaceutical Co.; WX provided standardized extract of BL02 for the whole study. All other authors declare that they have no competing interests.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.phrs.2019.104415.

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