The HLJ1-targeting drug screening identified Chinese herb andrographolide that can suppress tumour growth and invasion in non-small-cell lung cancer

Yi-Hua Lai1, Sung-Liang Yu2, Hsuan-Yu Chen3, Chi-Chung Wang4, Huei-Wen Chen5 and Jeremy J.W.Chen6,7,*

1Institute of Molecular Biology, National Chung Hsing University, Taichung, Taiwan, Republic of China, 2Department of Clinical Laboratory Sciences and Medical Biotechnology, National Taiwan University College of Medicine, Taipei, Taiwan, Republic of China, 3Institute of Statistical Science, Academia Sinica, Taipei, Taiwan, Republic of China, 4Institute of Basic Medicine, Fu Jen Catholic University, Taipei, Taiwan, Republic of China, 5Agricultural Biotechnology Center, National Chung Hsing University, Taichung, Taiwan, Republic of China and 6Institute of Biomedical Sciences, National Chung Hsing University, Taichung,Taiwan,Republic of China

*To whom correspondence should be addressed. Tel: +886 4 22840896 ext. 125; Fax: +886 4 22856549; Email: jwchen@dragon.nchu.edu.tw

Correspondence may also be addressed to Huei-Wen Chen. Tel: +886 2 23123456 ext. 86098; Fax: +886 2 22958341; Email: hwchen@ntu.edu.tw

HLJ1 is a novel tumour suppressor and is a potential druggable target for non-small-cell lung cancer (NSCLC). In this report, using a promoter-containing enhancer region as the HLJ1-targeting drug-screening platform, we identified several herbal compounds from a Chinese herbal bank with the capacity to enhance HLJ1 promoter activity and suppress tumour growth and invasion of NSCLC. Among the herbal drugs identified, the andrographolide (from Andrographis paniculata [Burn, f.] Nees.) most significantly induced HLJ1 expression and suppressed tumourigenesis both in vitro and in vivo. The andrographolide upregulates HLJ1 via JunB activation, which modulates AP-2α binding at the MMP-2 promoter and represses the expression of MMP-2. In addition, silencing of HLJ1 partially reverses the inhibition of cancer-cell invasion by andrographolide. Microarray transcriptomic analysis was performed to comprehensively depict the andrographolide-regulated signalling pathways. We showed that andrographolide can affect 939 genes (analysis of variance, false discovery rate < 0.05) that are dominantly involved in the diverse effects of andrographolide on anticancer invasion and proliferation. In conclusion, the HLJ1-targeting drug-screening platform is useful for screening of novel anticancer compounds. Using this platform, we identified andrographolide as a promising new anticancer agent that could suppress tumour growth and invasion in NSCLC.

Introduction

Lung cancer, predominantly the non-small-cell lung cancer (NSCLC) subtype, is the leading cause of cancer deaths worldwide (1). Thus, finding a new strategy to improve treatment outcome is a major challenge. The most common causes of death in lung cancer patients are treatment failure and metastasis, which also represents a major hindrance to effective therapy (2). Metastasis is a multi-step process that involves cell proliferation, migration, degradation of the basement membrane and invasion. Currently, there is no effective strategy to prevent or combat these metastatic processes. Therefore, developing novel compounds that target both tumour growth and metastasis is an important and urgent mission for the next generation of anticancer therapy research.

We have previously showed that HLJ1 was a tumour suppressor in NSCLC and correlated with patient survival (3). The HLJ1, also known as DNAJB4, is a member of the heat shock protein (HSP)-40 family and has shown promise in its dual anticancer effects on both inhibition of tumour growth and metastasis in NSCLC (3,4). Accumulated evidences support that HLJ1 is a potential biomarker and druggable target for NSCLC (3,5).

Screening drugs from traditional Chinese medicine have been suggested as a shortcut in searching for new leading compounds. Recently, several types of herbal compounds were proven to be potential anticancer drugs in vitro and in vivo (6). These compounds, including curcumin from ginger (7), epigallocatechin-3-gallate from green tea (8) and lycopene from tomato (9), could target certain important mechanisms in tumour growth and metastasis (6). These compounds have many advantages in cancer treatment. They are relatively safe and can target multiple diseases, improve radiotherapy and chemotherapy complications, decrease side effects or toxicities of cytotoxic agents and have great potential for clinical applications (10,11). Therefore, scientists have focused their attempts on setting up a screening platform to more efficiently identify potential compounds from traditional medicine (12,13).

Several high-throughput analysis platforms have been applied to accelerate the identification of new therapeutic strategies for anticancer metastasis, including methods relying on a high-content and image-based screen (14), a chemical microarray (15), a multiplex analysis of gene expression (16) and a luciferase reporter gene assay (17). For a specific target, the luciferase reporter assay is the most effective way to conduct preliminary drug screening.

In this study, using HLJ1 promoter and reporter assays, we established a high-throughput platform to screen Chinese herbs. We found that a natural product, andrographolide, can significantly induce HLJ1 expression and inhibit tumour growth in vitro and in vivo. The transcriptomic analysis using microarray technology and gene network prediction further revealed the possible molecular anticancer mechanisms of andrographolide in suppressing tumour growth and invasion.

Materials and methods

Cell culture and drug treatment

The human bronchial epithelial cell BEAS2B (ATCC CRL-9609) and the human lung adenocarcinoma cell lines CL1-5 (18), H358 (ATCC CRL-5807) and A549 (ATCC CCL-185) were cultured in Dulbecco’s modified Eagle’s medium (DMEM) (Gibco, Breda, The Netherlands) with 10% fetal bovine serum (FBS) (Gibco) containing 1% penicillin/streptomycin at 37°C in a humidified atmosphere of 5% CO₂. Andrographolide (purity 98%) was purchased from Sigma–Aldrich Chemical Co. and prepared at a concentration of 100mM in dimethyl sulphoxide (DMSO) as a stock solution. The working solution was freshly prepared by dilution with DMEM to desired concentrations. Controls received the same volumes of DMSO as did the experimental samples (i.e. final concentrations less than 0.1%).

Real-time reverse transcription–PCR and western blot analysis

The expression level of HLJ1 was determined using real-time RT–PCR on an ABI prism 7300 sequence detection system (Applied Biosystems, Foster, CA). TATA-box binding protein was used as the internal control (GenBank X54993). The detailed procedures and calculations have been described previously (19). Western blotting was used to examine the protein expression level of HLJ1 using anti-HLJ1 antibody (Developmental Studies Hybridoma Bank, University of Iowa, Iowa, USA) and antibuﬀer (Santa Cruz Biotechnology). The reaction was revealed with the ECL reagent (Amersham, Buckinghamshire, UK).
Fig. 1. Effects of andrographolide on HLJ1 expression. (A) Flowchart of drug screening. The criteria and procedures are described in the Supplementary method, available at Carcinogenesis Online. (B) The cytotoxic effect of andrographolide as determined by an MTT assay. The results are shown as percentages of the control response (0 μM). The IC_{50} of andrographolide was 20 μM, determined by MTT assay, and 0 μM represents 0.1% DMSO. (C) Transcriptional activity of the HLJ1 promoter with andrographolide treatment as determined by a reporter assay. Promoter activity was calculated as a ratio relative to the vehicle control (0.1% DMSO). (D) HLJ1 mRNA and protein expression as determined by real-time RT–PCR and western blotting. (E) Western blot analysis of HLJ1 in CL1-5
expression levels after andrographolide treatment. The detailed procedures were performed as described previously (20). The primary antibodies used for the western blot analyses included anti-FunD polyclonal and anti-funD monoclonal (1:500; Santa Cruz Biotechnology, Santa Cruz, CA), anti-Cyclin B1 monoclonal (1:1000; Santa Cruz Biotechnology), anti-p21 monoclonal (1:1000; R&D Systems, Minneapolis, MN), anti-CyclinD1 polyclonal (1:1000; Santa Cruz Biotechnology), anti-PRAP polyclonal (1:2000; Cell Signaling Technology, Danvers, MA), anti-caspase-3 polyclonal (1:1000; Chemicon International, Temecula, CA), anti-caspase-8 monoclonal (1:2000; Upstate Biotechnology, Lake Placid, NY) and anti-HLJ1 monoclonal (1:3000; Santa Cruz Biotechnology) antibodies. Monoclonal anti-β-tubulin and anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (1:5000; Upstate Biotechnology) antibodies were used as loading controls.

Transfection and luciferase reporter assays
All transfections were accomplished in triplicate in 24-well plates; a detailed protocol was described in previous studies (4,20). In the preliminary screening of Chinese herb medicines by our reporter gene assay, the pGL3-FRER′ plasmid (full-length HLJ1 promoter containing the enhancer element) was transfected into CL1-0 and CL1-5 cells using a modified calcium phosphate method (21). In the following experiments, a reporter assay was performed using Lipofectamine according to the manufacturer’s instructions (Invitrogen, Carlsbad, CA). The pGL3-FRER′, pGL3-FRER′-AP1m (AP-1 binding site mutation), pGL3-F2RER′ (without enhancer), pGL3-F41RER′ (basal promoter) or pGL3-p-Emi (minimal enhancer) plasmids were cotransfected with the control plasmid β-galactosidase construct (pSV-β-Gal; Promega, Madison, WI). The HLJ1-specific small interfering RNAs (siRNAs) and a scrambled control were transfected into CL1-5 cells using the RNAiFect transfection reagent (Qiagen, Hilden, Germany). The HLJ1-specific short hairpin (sh) RNAs (designated as pLKO.1-shHLJ1-A and pLKO.1-shHLJ1-B; TRCN0000022269 and TRCN0000002270) and luciferase shRNA (pLKO.1-shLuc; TRCN0000002244) lentiviral vectors were obtained from the National RNAi Core Facility (Academia Sinica, Taiwan) and then subjected to prepare lentivirus according to the standard protocol. Cells were infected with lentivirus at a multiplicity of infection of five in medium containing polybrene (8 μg/ml). After infection for 24 h, cells were treated with 2.5 μg/ml puromycin to select for puromycin-resistant pooled clones.

Cell viability and proliferation assay
A 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay was used to evaluate the cytotoxic effects or proliferative abilities of the tested herbal extracts or andrographolide for 48 h or various other durations as described previously (22). The viable cell numbers were directly proportional to the production of formazan following solubilization with DMSO. The absorbance at 570 nm (630 nm as the reference) was measured by spectrophotometric analysis, we performed SYBR Green real-time RT–PCR.

Colony formation assay
For the anchorage-dependent growth assay, 500 cells were resuspended in DMEM and seeded in six-well plates. After a week, the media were removed and the cells were washed and fixed with 4% paraformaldehyde. The fixed cells were stained with 0.05% crystal violet. For the anchorage-independent growth assay, the six-well plates were precoated with 0.1% agarose in DMEM supplemented with 10% FBS. CL1-5 cells were seeded at 1 × 10 3 cells per well in 0.35% agarose/DMEM with 10% FBS. After solidification, the cells in soft agar were treated with andrographolide. The plates were incubated for 2 weeks and then stained with 0.5 mg/ml p-iodonitrotetrazolium violet. Colonies with a diameter greater than 0.2 mm were counted under an inverted microscope. The assay was performed according to the procedures described previously (3).

Cancer-cell invasion and migration
A transwell membrane (8 μm pore size, 6.5 mm diameter; Corning Costar Corporation, MA) coated with or without Matrigel (2.5 μg/ml; BD Biosciences, San Jose, CA) was used for invasion and transwell migration assays as described previously (3). The upper wells were filled with serum-free medium and CL1-5 cells (3 × 10 5 or 5 × 10 5 cells per well). The lower wells of the transwells contained the same medium with 10% FBS. Migration capability was also assessed using the wound-healing approach described previously (25).

Gelatin zymography assay
Gelatinolytic activity was measured by gelatin zymography according to the method described previously (24). In general, the samples were prepared with 0.1% DMSO. The samples were not boiled before loading. The prepared samples were then subjected to electrophoresis on 8% SDS polyacrylamide gels containing 0.1% gelatin. After electrophoresis, the gels were washed on a shaker with distilled water containing 2.5% Triton X-100 for 30 min to remove SDS and then incubated in activation buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 100 mM NaF, 0.05% NaN 3 ) for 18–20 h at 37°C. The gels were stained with Coomassie brilliant blue R-250 followed by destaining with destain buffer.

Flow cytometry and apoptosis
The CL1-5 cells were seeded at a density of 2 × 10 4 cells/60 mm dish in medium with 10% FBS and treated with andrographolide for 48 h. Each sample was washed with ice-cold phosphate-buffered saline, trypsinized, harvested and fixed in 75% (vol/vol) ethanol for 30 min at 20°C. The cells were then treated with RNase A at 37°C and stained with 25 μg/ml propidium iodide or annexin V/PI. These samples were analysed by flow cytometry using a Cytomics FC500 flow cytometer (Beckman Coulter, Fullerton, CA) according to the manufacturer’s protocol.

Oligonucleotide microarray analysis
The complementary RNA preparation and array hybridization were performed according to the Affymetrix GeneChip Expression Analysis Technical Manual. All hybridization experiments were performed in triplicate with complementary RNA probes prepared from CL1-5 cells treated with or without 10 μM andrographolide. The microarray data were filtered by 2-fold changes using Genespring GX 11 (Silicon Genetics, Redwood, CA). The detailed procedures have been described previously (3). The statistical analysis logic and algorithms used are described in the Affymetrix manual. To confirm the results derived from the microarray analysis, we performed SYBR Green real-time RT–PCR.

In vivo tumorigenesis
Six week old severe combined immunodeficiency (nude) mice (supplied by the animal centre of the College of Medicine, National Taiwan University, Taipei, Taiwan) were housed with six mice per cage. We achieved tumour growth in the mice according to protocols described previously (3). In total, 1 × 10 5 live CL1-5 cells were calculated and injected subcutaneously into the nude mice. To examine the effects of andrographolide on tumour growth, the mice were grouped into andrographolide-treated or untreated groups. The treated cohort was treated with andrographolide at 4 mg/kg once daily for six weeks. After CL1-5 injection, the mice were examined every 5 days for tumour appearance. The tumour volumes were estimated from their caliper-measured lengths (a) and widths (b) using the formula V = 0.4 × a × b 2 (25). After 6 weeks, the mice were killed and their tumour sizes were analysed. The mouse experiments were approved by the Institutional Animal Care and Use Committee of the National Chung Hsing University.

Chromatin immunoprecipitation assay
Chromatin immunoprecipitation (ChIP) was performed according to the manufacturer’s instructions (Upstate Biotechnology). The CL1-5 cells were cross-linked in a 1% formaldehyde solution for 10 min at 37°C. After sonication and centrifugation, the cleared supernatant was diluted with ChIP buffer and incubated with the indicated antibodies at 4°C. Immune complexes were precipitated, washed and eluted as recommended by the manufacturer. DNA–protein cross-links were reversed by heating at 65°C. Subsequently, DNA fragments were purified and dissolved in water, followed by PCR amplification with MMP-2 and GAPDH promoter primers as described previously (23).

Statistical analysis
The results are presented as the mean ± standard deviation, and all experiments were performed at least in triplicate. All data were analysed for significant differences by analysis of variance (Excel; Microsoft). P values of less than 0.05 were considered statistically significant.

Results
Chinese herbal medicine screening via the HLJ1-targeting drug-screening platform
The criteria and procedures used in our drug screen are illustrated in Figure 1A and described in the Supplementary method, available at Carcinogenesis Online. The MTT assay was performed to determine
Fig. 2. HLJ1 upregulation by andrographolide via AP-1. (A) Transcriptional activity of the HLJ1 promoter in CL1-5 cells with 10 μM andrographolide (Andro) treatment as determined by reporter assay. The promoter constructs, including the deleted enhancer region (pGL3-F2RER') and the basal promoter (pGL3-F41RER'), are illustrated in the left panel. (B) The effect of the AP-1 binding motif on HLJ1 transcriptional activity. The results indicated that the AP-1 site was crucial for andrographolide-induced HLJ1 enhancer activity. The promoter constructs, including full-length (pGL3-FRER') and AP-1 mutation (pGL3-FRER'-AP1m), are illustrated in the left panel. (C) Transcriptional activity of the HLJ1 minimum enhancer in CL1-5 cells as determined by reporter assay. The Sp1 site mutant (Emi-SPF/R) has no significant effect on reporter activity compared with the wild-type (Emi-F/R). Top: AP-1 and Sp1 mutation constructs. (D) Western blot analysis of JunB and JunD in CL1-5 cells after andrographolide treatment. GAPDH was used as a loading control. Each treatment was independently performed in triplicate. *P < 0.05 compared with the control (0 μM).
Fig. 3. Suppression of cancer cell proliferation and tumour growth by andrographolide. (A) Cell proliferation analysis as detected by MTT assay. The CL1-5 cells were treated with the indicated concentrations of andrographolide for 0, 24, 48 or 72h. (B) Anchorage-dependent colony formation assay. CL1-5 cells grown in a culture dish without soft agar were treated with the designated concentrations of andrographolide. (C) Anchorage-independent colony formation assay. CL1-5 cells grown in soft agar were treated with the designated concentrations of andrographolide and then subjected to colony formation analyses. *P < 0.05
the antiproliferation activity of the 90 herbal extracts (Supplementary Table S1, available at Carcinogenesis Online), followed by screening with the promoter assay and western blot validation for HLJ1 induction. In accordance with such criteria, the extract from Andrographis paniculata (Burm.f.) Nees was identified as the best candidate for this study (Supplementary Figure S1–5, available at Carcinogenesis Online). Consequently, we focused on andrographolide, the major diterpenoid lactone constituent of Andrographis paniculata (Burm.f.) Nees.

In accordance with such criteria, the extract from Andrographis paniculata (Burm.f.) Nees. was identified as the best candidate for this study (Supplementary Figure S1–5, available at Carcinogenesis Online). Consequently, we focused on andrographolide, the major diterpenoid lactone constituent of Andrographis paniculata, which has been reported to have numerous pharmacological activities (26-28).

Promotion of HLJ1 expression by andrographolide

The cytotoxic assay indicated that cell viability was not significantly influenced by andrographolide at 10 μM, a dose that is approximately equivalent to IC_{50}. However, andrographolide exhibited cytotoxicity at the concentration over 20 μM compared with the solvent control (0.1% DMSO; IC_{50} ≈ 40 μM, Figure 1B). To determine whether andrographolide had similar effects as Andrographis paniculata on HLJ1 expression by regulating the HLJ1 promoter, a luciferase reporter assay was performed. The results confirmed that the luciferase activity triggered by the HLJ1 promoter is enhanced in a concentration-dependent manner by andrographolide (5–20 μM; Figure 1C). Real-time PCR and western blots for HLJ1 demonstrated that andrographolide induces HLJ1 messenger RNA (mRNA) and protein expression (Figure 1D). Similar results were obtained in another lung cancer cell lines, A549 and H358 (Supplementary Figure S6A and B, available at Carcinogenesis Online). Furthermore, we determined that andrographolide induced HLJ1 protein expression in a time-dependent manner, ranging from a few hours (Figure 1E) to 5 days (Figure 1F). However, considering the cytotoxic effect of 20 μM andrographolide on cell viability, for all experiments of cellular functions other than cell cycle and apoptosis analyses, we used less cytotoxic concentrations of no more than 10 μM, which are safe concentrations for cell growth in this study.

HLJ1 upregulation by andrographolide via JunB

Previously, we identified that YY1 and AP-1 (JunB and JunD) are key regulators of HLJ1 transcriptional activity (4,20). To explore whether andrographolide is able to regulate HLJ1 through its promoter and/or enhancer regions, various constructs with mutations were used as described previously (4). The results in Figure 2A demonstrate that compared with the vehicle-treated control. Each treatment was independently performed in triplicate. (D) In vivo tumorigenesis. One million CL1-5 cells were subcutaneously injected into nude mice. These mice were then treated with or without andrographolide (Andro). Tumours were examined every 5 days for tumour appearance and tumour volumes were measured by callipers. Right: representative photographs of primary tumours from the subcutaneous tissue of SCID mice. The tumour weights are presented as the mean ± standard deviation of six mice per group. *P < 0.05 compared with the untreated control.
Fig. 4. Effects of andrographolide on cell cycle and apoptosis. (A) Analysis of cell-cycle progression. CL1-5 human lung cancer cells were treated with andrographolide and then subjected to flow cytometry. Upper panel: the representative image of the flow cytometry analysis. Lower bar chart: the average of triplicate experiments. *P < 0.05 compared with the control (0 μM). (B) Western blot analyses of Cyclin B1, Cyclin D1 and p21 in CL1-5 cells treated with the designated concentrations of andrographolide. (C) Apoptotic analysis. The CL1-5 cells were treated with varying concentrations of andrographolide for 48 h. The apoptotic cells were stained with Annexin V and examined by flow cytometry. (D) Western blot analyses of HLJ1, PARP, Casp-8, and Casp-3 in CL1-5 cells treated with the designated concentrations of andrographolide. GAPDH served as a loading control.
the luciferase activity could not be upregulated by andrographolide in the absence of enhancer. Indeed, activity was even downregulated (pGL3-F2RER), indicating the importance of the enhancer region in andrographolide-induced HLJ1 expression.

To further address this issue, the HLJ1 promoter construct with an AP-1 mutation (pGL3-FRER-AP1m) in the enhancer region was used to demonstrate that andrographolide is able to enhance HLJ1 promoter activity, most probably through AP-1 (Figure 2B).

Furthermore, andrographolide significantly enhanced the luciferase activity of the minimum enhancer region (Emi-F/R), which included the AP-1 and Sp1 binding sites. Site-directed mutagenesis demonstrated that the AP-1 site (1457–1451) but not the Sp1 site may contribute significantly to andrographolide-regulated HLJ1 enhancer region activity (Figure 2C). The effects of andrographolide on the AP-1 family indicated that only JunB can be significantly induced by andrographolide in CL1-5 cells; JunD was slightly downregulated by andrographolide over the range of concentrations used (Figure 2D).

Suppression of cancer-cell proliferation and tumour growth by andrographolide

The cell proliferation, anchorage-dependence and in vivo tumour growth model assays were performed to investigate the anticancer effects of andrographolide. Our results demonstrated that andrographolide could inhibit CL1-5 cell proliferation in a time-dependent manner (0, 24, 48 and 72h; Figure 3A). Similar results were also observed in A549 cells (Supplementary Figure S6C, available at Carcinogenesis Online). Herein, we showed that the colony formation of CL1-5 cells was inhibited by andrographolide in a concentration-dependent manner (1, 3 and 5 μM) within weeks irrespective of anchorage-dependent (Figure 3B) or anchorage-independent growth (Figure 3C). Significantly, colony formation capability was totally suppressed by 5 μM andrographolide treatment.

To further evaluate the antitumour effect of andrographolide in vivo, CL1-5 cells were injected subcutaneously into nude mice. These mice were then randomly grouped and treated with or without andrographolide (p.o., 4 mg/kg/day). Andrographolide treatment produced no severe adverse events as monitored by animal survival. Tumour sizes were significantly reduced in the andrographolide-treated group, whereas the control group exhibited continuous growth (Figure 3D, left). After 5 weeks, the animals were killed to investigate tumour weights. Significantly reduced tumour growth was observed in the andrographolide treatment group, with an average size of 72 mm³ (95% CI = 17–180 mm³; P = 0.01) compared with the control group with an average of 522 mm³ (95% CI = 440–950 mm³) (Figure 3D). The tumour weights were also significantly decreased from 549 mg to 120 mg (P = 0.0004; Figure 3D, right).

Andrographolide-induced transcriptomic analysis and gene networks

To further determine the mechanisms by which andrographolide inhibits cell proliferation and invasion, an Affymetrix oligonucleotide microarray was performed. A total of 939 genes exhibiting significant alterations after andrographolide treatment were identified by analysis of variance (FDR < 0.05). According to the pathway analysis by the CRSID web server made in-house (29) and GeneGo (http://www.genego.com/metacore), the major regulatory pathways involved in the mechanisms of andrographolide effects included cell cycle, apoptosis, regulation of actin cytoskeleton, focal adhesion, tight junction and mitogen-activated protein kinase pathways. The andrographolide-regulated genes involved in these pathways were verified by SYBR green real-time RT–PCR (Table I).

Inhibition of cell-cycle progression in lung cancer cells by andrographolide

Based on the pathway prediction and real-time RT–PCR validation, we focused on the cell-cycle regulatory pathway as the major target for andrographolide. We determined that 20 μM of andrographolide can slightly reduce the ratio of the S phase population from 13.28 ± 0.379% (control) to 10.48 ± 1.824% and that 20 μM of andrographolide can significantly increase the ratio of the sub-G0/G1 population from 3.07 ± 0.242% (control) to 13.79 ± 1.539% when cells were treated for 48 h. Additionally, high-dose (20 μM) andrographolide treatment arrested CL1-5 cells at the G2/M phase (40.32 ± 0.861%) compared with the 0.1% DMSO control (26.38 ± 0.301%; Figure 4A). Furthermore, coincident with G2/M arrest, the expressions of Cyclin B1 and p21 in addition to HLJ1 were increased in CL1-5 cells after andrographolide treatment (Figure 4B). In contrast, Cyclin D1 was reduced with increasing concentrations of andrographolide. The results demonstrate that andrographolide can inhibit CL1-5 cell proliferation by inhibiting cell-cycle progression.

Apoptotic induction of lung cancer cells by andrographolide

Figure 4A demonstrates that 20 μM andrographolide can increase the population of sub-G0/G1 phase CL1-5 cells. To study whether andrographolide can cause apoptosis, CL1-5 cells were treated with varying concentrations (0, 5, 10 and 20 μM) of andrographolide. After 48 h of treatment, apoptosis induction by andrographolide was examined by annexin V/PI staining. The results demonstrated that andrographolide induced apoptotic cell death in a concentration-dependent manner, with up to a 2.6-fold increase in apoptotic cell death at 20 μM (Figure 4C). Western blotting also provided evidence that the typical 87 kDa poly (ADP ribose) polymerase (PARP) cleavage product was significantly increased by andrographolide treatment. In addition, both caspase-8 and -3 activities were markedly increased in CL1-5 cells with 48 h of andrographolide treatment (20 μM; Figure 4D).

The role of HLJ1 in the anti-invasive effects of andrographolide

Our previous report showed that HLJ1 can inhibit cancer-cell invasion and migration (3). The transcriptomic analysis of this study also revealed that andrographolide can regulate certain metastasis-related factors (Table I). To investigate the effects of andrographolide on invasion and migration, CL1-5 cells were pre-treated with varying concentrations of andrographolide for 48 h and then subjected to invasion and migration assays for 24 h and 12 h, respectively. Our data demonstrated that andrographolide significantly inhibits cell migration and invasion, especially at the concentration of 10 μM (Figure 5A and B). In addition, HLJ1-specific siRNA was employed to explore the role of HLJ1 in the anti-invasive effects of andrographolide. The results indicated that andrographolide-induced HLJ1 was knocked down by approximately 50%; coincidentally, the anti-invasive effects of andrographolide were diminished by pretreatment with HLJ1 siRNA (Figure 5C). In addition to invasiveness, we also found that silencing HLJ1 would render cancer cells more resistant to andrographolide treatment compared with the shLJ1 control (Supplementary Figure S7, available at Carcinogenesis Online). Moreover, the antiproliferative effects of andrographolide were reduced by silencing HLJ1 compared with the negative control (Supplementary Figure S8, available at Carcinogenesis Online).

Extracellular matrix degradation is an important aspect of cancer-cell invasion and usually involves matrix-degrading proteinases (30). Our gelatin zymography revealed that MMP-2 activity was decreased by andrographolide in a concentration-dependent manner (Figure 5D). In addition, real-time RT–PCR demonstrated that andrographolide also significantly reduced the mRNA levels of MMP-2 to approximately...
Fig. 5. The role of HLJ1 in the anti-invasive effects of andrographolide. (A) Effects of andrographolide on cell invasion as analysed by a Matrigel invasion assay. *P < 0.05 compared with the control (0 μM). (B) Suppression of cell migration by andrographolide as determined using transwell assay for 12 h. *P < 0.05
42% of that of the control (Figure 5E). Our previous study also demonstrated that HLJ1 may downregulate MMP-2 by forming a protein complex with NPM1 and the suppressor AP-2 (23). To clarify whether the repressive effect of andrographolide on MMP-2 is mediated through the formation of an AP-2α complex, a ChIP-PCR assay using an anti-AP-2α antibody was performed. The AP-2α-binding consensus region in the promoter of MMP-2 is illustrated in Figure 5F (top). The results indicated that the interaction between the MMP-2 promoter and the AP-2α is enhanced in the CL1-5 cells treated with andrographolide compared with the control (Figure 5F, bottom).

Discussion
To screen for potential agents among traditional Chinese herbs that can target the novel onco-suppressive gene HLJ1 (3), the present study developed an HLJ1-targeting drug-screening platform to screen for anticancer compounds and also revealed a role for andrographolide in cancer growth and metastasis inhibition in vitro and in vivo. Andrographolide, a diterpenoid lactone isolated from the Chinese herbal medicine Andrographis paniculata, is known for its wide pharmacological activities, such as its anti-inflammation, anti-angiogenesis, pro-apoptosis and anticancer activities (26,27). Moreover, Andrographis paniculata has long been perceived as safe in traditional Chinese medicine and in the systems of traditional medicine of Thailand and India (31,32). The previous reports indicated that Andrographis paniculata is genotoxically safe (33) and has been applied to clinical investigations of the treatment and prevention of upper respiratory tract infections (34).

In this study, the non-tumorigenic human bronchial epithelial cells (BEAS2B) also showed the lower cytotoxicity (IC₅₀ = 44 μM) than the human lung adenocarcinoma cell lines CL1-5 (IC₅₀ = 25 μM) and A549 (IC₅₀ = 22 μM) after treatment. However, the mechanisms of action associated with andrographolide and YY1 are still unclear. This is the first study to demonstrate that andrographolide can transcriptionally activate HLJ1 expression through JunB of the AP-1 family to further inhibit cancer-cell proliferation, migration and invasion. Our results also establish the potential of andrographolide as a multi-target lead compound in developing anticancer therapies (28).

Our previous study confirmed that the transcription factors AP-1 and YY1 can bind to the enhancer and promoter regions, respectively, of the tumour-suppressor HLJ1 to upregulate its expression (4). In this study, regardless of whether the HLJ1 promoter used was a full-length promoter or a minimal enhancer, all of the reporter assays revealed that andrographolide-induced HLJ1 promoter activity was mediated by AP-1 and not by YY1 or Sp1. We determined that JunB is the major protein that enhances HLJ1 expression after andrographolide treatment. Based on our previous studies (3) and the current results, we suggested that andrographolide could inhibit cell proliferation, anchorage-dependent/independent growth and tumorigenesis of lung cancer cell by upregulating HLJ1. These results also indicated that andrographolide might be a potential targeted anticancer therapy compound that enhances HLJ1 expression.

However, the question remained whether HLJ1 is the only anticancer target of andrographolide. The downstream gene analysis using microarray technology indicated that andrographolide can modulate many genes and related pathways, including cell cycle, apoptosis, mitogen-activated protein kinase, focal adhesion and tight junction pathways. It has been reported previously that andrographolide can induce p53 and p27 while decreasing CDK4 expression to cause a G₁/G₀ or G₀/M phase arrest in human colorectal carcinoma cells (35). Additionally, andrographolide can induce a decrease in the population of Hep3B cells in the G₁/G₀ phase and a concomitant accumulation of cells in the G₀/M phase by downregulating the expression of Cyclin D1, Cdc-2 and phosphorylated-Cdc-2 (36). The cell cycle–related genes that were identified by our microarray analysis included Cyclin B1, BUB1, Cdc25c, GADD45B and CCND3. Cyclin B1 is a checkpoint protein that regulates mitotic entry in many cell types. Several studies have established that certain anticancer drugs can increase Cyclin B1 accumulation and induce G₀/M phase arrest in human cancer cell lines (37,38). Budding inhibited by benzimidazole 1 (BUB1) plays a central role in the spindle assembly checkpoint. Hence, the deletion of BUB1 from Saccharomyces cerevisiae leads to slow growth and elevated chromosome loss (39). The spindle assembly checkpoint machinery, including the kinase BUB1, prevents cells entering anaphase by inactivating Cdc20 and inhibiting the anaphase promoting complex/cyclosome ubiquitin ligase to recognize cyclin B1 and securin (40,41). In addition, the expression levels of p21 and Cyclin D1 after andrographolide treatment are consistent with our previous study involving the over-expression of HLJ1 (3). Consequently, it is reasonable to speculate that andrographolide regulates cell-cycle progression by enhancing HLJ1 expression and increasing the cell cycle–related gene expression of Cyclin B1 and BUB1.

Our microarray and pathway analyses also revealed that andrographolide can induce cell apoptosis in NSCLC cells. The apoptosis pathway implies caspase activation, which is stimulated in a proteolytic cascade to cleave specific substrates such as PARP (42). Caspase activation is specifically initiated through two major apoptotic mechanisms: mitochondrial death and receptor-mediated cell-death pathways. The mitochondrial death pathway follows the release of cytochrome c from the mitochondria and activates the downstream effector caspase-3 after caspase-8 activation (43). Previous studies reported that andrographolide induces cell apoptosis via the mitochondrial pathway and activates caspase-8 and caspase-3 in liver cancer, cervical cancer, breast cancer and prostate cancer cells (44–46). Our results suggest that andrographolide treatment can increase HLJ1 expression and further induce BID expression, PARP cleavage, and caspase-8 and caspase-3 activations in NSCLC cells, ultimately leading to increased apoptosis.

Cancer-cell metastasis occurs over a series of steps, including cell adhesion, migration, invasion and angiogenesis. These steps are regulated by exceptionally intricate mechanisms (47). Matrix metalloproteinase (MMP) activation is an important part of this mechanism because it plays a key role in promoting tumour invasion (30). Previously, andrographolide was reported to inhibit colorectal cancer cell invasion and migration by suppressing the activity of c-Fos and c-Jun and thus reducing MMP-7 expression (48). Andrographolide was also reported to suppress invasion and migration in A549 lung cancer cells through attenuation of the PI3K/Akt signalling pathway (49). Here, we demonstrated that andrographolide inhibits CL1-5 lung cancer cell invasion and migration through the upregulation of
Andrographolide inhibits cancer-cell progression via HLJ1

HLJ1 and the downregulation of MMP-2 expression and activity. Our previous study indicated that HLJ1 can regulate MMP-2 activity and expression via NPM1 as an AP-2 co-repressor (23). Moreover, previous evidence had demonstrated that AP-2 proteins can regulate MMP-2, E-cadherin and p21WAF-1 in human melanoma cells (50). Based on this evidence, we confirmed that HLJ1 indeed enhances AP-2α binding to the MMP-2 promoter in CL1-5 lung cancer cells via a chromatin immunoprecipitation assay. These results suggested that andrographolide inhibits cancer-cell invasion by increasing HLJ1 expression and AP-2α activation to decrease MMP expression and activity.

In conclusion, we established an HLJ1-targeting drug-screening platform that can be employed to screen a large number of traditional Chinese herbal medicines. Furthermore, we discovered novel pathways associated with the ability of andrographolide to suppress tumorigenesis. The onco-suppressive effects of andrographolide may be partially mediated via JunB-regulated HLJ1 expression pathways associated with the ability of andrographolide to suppress tumorigenesis. These results suggest that the HLJ1-targeting drug-screening platform is a valuable tool and that andrographolide has the potential to be an important compound in anticancer drug development.

Supplementary material

Supplementary Table 1 and Figures S1–S8 can be found at http://carcin.oxfordjournals.org/

Funding

National Research Program for Biopharmaceuticals grant from the National Science Council, Taiwan, R.O.C. (NSC 100-2325-B-005-001 and NSC 101-2325-B-005-001); Ministry of Education, Taiwan, R.O.C. under the ATU plan.

Acknowledgements

The shRNA constructs were obtained from the National RNAi Core Facility located at the Institute of Molecular Biology/Genomic Research Center, Academia Sinica. We thank the Microarray Core Facility of National Taiwan University Center of Genomic Medicine for collaboration and technique assistance.

Conflict of Interest Statement: none declared.

References


Received July 28, 2012; revised December 16, 2012; accepted January 3, 2013