Jacarelhyperol A induced apoptosis in leukaemia cancer cell through inhibition the activity of Bcl-2 proteins

Shoude Zhang1,2, Jun Yin2, Xia Li1, Jigang Zhang1, Rongcai Yue1, Yanyan Diao2, Honglin Li2, Hui Wang3, Lei Shan1* and Weidong Zhang1,2*

Abstract

**Background:** *Hypericum japonicum* Thunb. ex Murray is widely used as an herbal medicine for the treatment of hepatitis and tumours in China. However, the molecular mechanisms of its effects are unclear. Our previous research showed that extracts of *H. japonicum* can induce apoptosis in leukaemia cells. We also previously systematically analysed and isolated the chemical composition of *H. japonicum*.

**Methods:** The fluorescence polarisation experiment was used to screen for inhibitors of Bcl-2 proteins which are proved as key proteins in apoptosis. The binding mode was modelled by molecular docking. We investigated the proliferation attenuating and apoptosis inducing effects of active compound on cancer cells by MTT assay and flow cytometry analysis. Activation of caspases were tested by western blot. A broad-spectrum caspase inhibitor Z-VAD-FMK was used to investigate the caspases-dependence. In addition, co-immunoprecipitation was performed to analyse the inhibition of heterodimerization between anti-apoptotic Bcl-2 proteins with pro-apoptotic proteins. Moreover, in vivo activity was tested in a mouse xenograph tumour model.

**Result:** Jacarelhyperol A (Jac-A), a characteristic constituent of *H. japonicum*, was identified as a potential Bcl-2 inhibitor. Jac-A showed binding affinities to Bcl-xL, Bcl-2, and Mcl-1 with $K_i$ values of 0.46 μM, 0.43 μM, and 1.69 μM, respectively. This is consistent with computational modelling results, which show that Jac-A presents a favorable binding mode with Bcl-xL in the BH3-binding pocket. In addition, Jac-A showed potential growth inhibitory activity in leukaemia cells with IC$_{50}$ values from 1.52 to 6.92 μM and significantly induced apoptosis of K562 cells by promoting release of cytochrome c and activating the caspases. Jac-A also been proved that its effect is partly caspases-dependent and can disrupt the heterodimerization between anti-apoptotic Bcl-2 proteins with pro-apoptotic proteins. Moreover, Jac-A dose-dependently inhibited human K562 cell growth in a mouse xenograph tumour model with low toxicity.

**Conclusion:** In this study, a characteristic constituent of *H. japonicum*, Jac-A, was shown to induce apoptosis in leukaemia cells by mediating the Bcl-2 proteins. Therefore, we propose a new lead compound for cancer therapy with a low toxicity, and have provided evidence for using *H. japonicum* as an anti-cancer herb.

**Keywords:** Bcl-2, Jacarelhyperol A, Apoptosis, *Hypericum japonicum* Thunb ex Murray, Leukemia
Background
The entire Hypericum japonicum herb, named “Tianjihuang,” is widely used for the treatment of infectious hepatitis, acute and chronic hepatitis, and tumour in China [1]. An 85% ethanol-treated water extract is documented in the Chinese Pharmacopoeia as an injection for the treatment of viral hepatitis [2,3]. Moreover, H. japonicum is used as an animal feed in China because of its widespread growth. These records demonstrate the clinical safety of H. japonicum. However, the molecular mechanisms of its effects are unclear. To better understand the mechanisms of H. japonicum, its chemical composition was systematically isolated and analysed in our previous study. In this study, we identified jacarehyperol A (Jac-A), a characteristic constituent of H. japonicum, as a potent inhibitor of Bcl-2 proteins via high throughput screening of an in-house natural product library (NPL).

The Bcl-2 family of proteins play an important role in apoptosis through the balance of antiapoptotic proteins (e.g., Bcl-2, Bcl-xL, Mcl-1) and proapoptotic proteins (e.g., Bak, Bax, Bad, Bid) [4]. The ability of antiapoptotic proteins to form heterodimers with a number of proapoptotic proteins is believed to play a crucial role in their antiapoptotic function [5]. Antiapoptotic Bcl-2 proteins are overexpressed in a variety of tumours, which can protect cancer cells from apoptosis [6,7]. Owing to their important functions in regulating cell death, the pharmacological inhibition of Bcl-2 proteins is a promising strategy for apoptosis induction or sensitisation to chemotherapy [8]. Protein secretion of Bcl-2 proteins is a promising strategy for apoptosis through the balance of antiapoptotic proteins and their antiapoptotic Bcl-2 family members.

Methods
Fluorescence polarisation assay
The Bid BH3 domain peptide (sequence: EDIIPNIAHR-LAQVGDSDMR) has been identified as the BH3 domain, which is the fundamental motif for the dimerisation with antiapoptotic proteins [9]. The three-dimensional structure of complex of Bcl-xL and the Bak BH3 domain peptide showed that the Bid peptide is an amphipathic α-helix that passes to a hydrophobic groove on the surface of Bcl-xL [10]. Based on these studies, screening new ligands at hand to the same pocket became an anti-cancer drug discovery strategy to search for antiapoptotic protein inhibitors [11]. To screen for Bcl-2 protein inhibitors, we used fluorescence polarisation (FP), whose basic principle is that a fluorescent peptide tracer (Flu-Bid-BH3) and a non-fluorescent small molecule inhibitor compete for binding to the Bid BH3 domain of Bcl-2 proteins. Jac-A was chosen as the candidate compound for further research because of its high affinity with Bcl-xL and favourable binding mode with Bcl-xL. Then, we tested its anti-cancer activity in vitro and in vivo. Jac-A possesses a broad antitumour effect for all tested cancer cells and remarkably inhibited the proliferation of leukaemia cells. Moreover, Jac-A not only induced K562 cell apoptosis in vitro, but also inhibited human K562 cell growth in a mouse xenograph tumour model, which provided evidence for using H. japonicum as an anti-cancer herbal medicine. We also proved that Jac-A’s effect is partly caspase-dependent and it can disrupt the heterodimerization between anti-apoptotic Bcl-2 family members with pro-apoptotic Bcl-2 family members.

Molecular modelling
The refined structure of Bcl-xL (PDB: 2YXJ) was used for prediction binding mode between Jac-A with Bcl-xL. The program Maestro 9.0 was used for this assessment. All water molecules were removed from the structure of the complex. Hydrogen atoms and charges were added during a brief relaxation that was performed using the “Protein Preparation Wizard” workflow in Maestro 9.0. After optimising the hydrogen bond network, the crystal structure was minimised using the OPLS 2005 force field with the maximum root mean square deviation (RMSD) value of 0.3 Å. The grid-enclosing box was centred on the ligand ABT-737 in the refined crystal structure as described above, and defined so as to enclose the residues located within 14 Å from the ligand. This domain has been identified as the BH3 domain, which is the fundamental motif for dimerization with the BH3 peptide. The three-dimensional structure of Jac-A was generated with the Ligprep module. Docking process was performed using GLIDE with default docking parameter setting with extra precision (XP) approach.

Cell culture
Cell lines MBA-MB-231, T47D, LOVO, A549, HepG2, K562, HL-60, and THP-1 cells were obtained from the American Type Culture Collection (Manassas, VA). All cell culture supplies were obtained from Invitrogen (Carlsbad, CA). Thiazolyl blue tetrazolium bromide (catalogue no. M5655) and dimethyl sulfoxide (catalogue no. D5879) were purchased from Sigma-Aldrich (St. Louis, MO). Cells were cultured in RPMI 1640 (A549, K562,
Cytotoxicity assay

The cytotoxic activity of Jac-A against human cancer cells was measured by the MTT colorimetric assay. Four thousand cells (per well) were seeded in 96-well plates and treated with the compounds for 48 h at serial concentrations. Then, 10 μL MTT solution (5 mg/mL in PBS) was added to each well, and the plates were incubated for an additional 2–4 h at 37°C. The supernatant was carefully removed, and 100 μL DMSO was added to dissolve the formazan crystals. The absorbance at 570 nm was recorded on a BioTek Synergy 2 plate reader (BioTek Instruments, Inc., Winooski, VT, USA).

Detection of apoptosis by flow cytometry using Annexin V-PI staining

After treated with 0 (control), 0.1, 1, 5, 10 μM/L Jac-A and 0.5% DMSO for 48 h, K562 cells from each group were collected and diluted to a concentration of 1.0 × 10⁶ per mL. The cells were washed with cold PBS twice and resuspended in 100 μL Annexin-V-FITC (Sigma) diluted 1:100 in binding buffer (10 mM Hepes 100 mM NaCl, 1 mM KCl, 1 mM MgCl₂, 1.8 mM CaCl₂) containing 1% protease inhibitor cocktail (Thermo Scientific). Fifty micrograms protein per lane was electrophoresed on 10% SDS polyacrylamide gels. Nonspecific reactivity was blocked by 5% non-fat milk prepared in TBST (10 mM Tris, 150 mM NaCl, 0.05% Tween-20, pH 7.5) at room temperature for 1 h. The membranes were incubated with antibodies diluted according to the manufacturers’ instructions. Images were captured by the Odyssey infrared imaging system (LI-COR Bioscience, Lincoln, NE). Protein densitometry was performed with the Odyssey infrared imaging software (LI-COR) and normalised against β-actin. Antibodies for cleaved PARP, PARP, cleaved caspase-9, caspase-9, cleaved caspase-3, caspase-3, and β-actin were obtained from Cell Signaling Technology (Beverly, MA).

Cytochrome c release assay

The method of preparing mitochondrial cytosol was referenced to others [13-15]. Briefly, after treated with 0 (control), 3, 6, 12 μM/L Jac-A for 48 h, K562 cells (1 × 10⁶) were collected and washed once with ice-cold PBS and re-suspended in a mitochondrial isolation buffer (250 mM sucrose, 20 mM HEPES, pH 7.4, 5 mM MgCl₂ and 10 mM KCl) containing 0.05% digitonin. Cells were left on ice for 10 h, followed by centrifugation at 13000 r.p.m. for 3 min. The pellet was the mitochondrial membrane (heavy membrane proteins) portion. Soluble fraction proteins and an equivalent amount of heavy membrane proteins were subjected to SDS-PAGE and analysed by Western blot with antibodies against Cyt c (Abcam, CA).

Caspase activation assay by western blotting

After treated with 0 (control), 3, 6, 12 μM/L Jac-A for 48 h, K562 cells (1 × 10⁶) were collected and suspended in lysis buffer containing 150 mM NaCl, 10 mM HEPES [pH 7.4], 1% CHAPS, 1% NPM, 5 μg/mL leupeptin, 5 μg/mL aprotinin and 1 μg/mL pepstatin A. 150 μg of K562 cell lysates in 500 μL of CHAPS lysis buffer were precleared for 60 min at 4°C and washed with 20 μL of a 1:1 slurry of protein A/G Plus-Agarose (Santa Cruz Biotechnology, Cat. sc 2003) and 0.01 μg of rabbit IgG. After a brief centrifugation (3000 × g for 5 min at 4°C) to remove precleared beads, 1 μg of rabbit anti-Bax or Bakpolyclonal antibody and 20 μL of Protein G Plus-Agarose were added to the lysate, followed by incubation at 4°C overnight on a rotating device. Precipitates were washed four times with CHAPS buffer, re-suspended in 30 μL 1× SDS electrophoresis sample buffer (50 mM Tris–HCl (pH 6.8), 100 mM dithiothreitol, 2% SDS, 0.1% bromophenol blue, and 10% glycerol), electrophoresed, and analysed by Western blotting with monoclonal antibodies against Bcl-xL, Bcl-2, Mcl-1, Bax, and Bak, respectively. All antibodies in this experiment were purchased from Abcam (Shanghai, CA).

Xenograph tumor model in mice

Female Balb/c nude mice (5 weeks old) were purchased from Shanghai SLAC Laboratory Animal Co., LTD (Shanghai, China). 5 × 10⁶ K562 cells were subcutaneously injected in the right flank of mice. When the tumours reached approximately 200 mm³, the mice were randomly divided into four groups (n = 10 mice/each group) and treated with Jac-A at 2, 10, 50 mg/kg or vehicle by oral gavage. Tumour growth was monitored by measuring the tumour size twice a week for 3 weeks after treatment. A digital caliper was used to measure the tumour in two orthogonal dimensions. The volume was calculated with the formula (long dimension) × (short dimension)²/2. The body weight and survival of the nude mice were monitored throughout the experiments. All animal experiments
were approved by the animal care committee of the Second Military Medical University in accordance with institutional and Chinese government guidelines for animal experiments.

Statistical analysis
The data from the in vitro and in vivo experiments at different time points for the different treatment groups were analysed for statistical significance with the GraphPad Prism program (GraphPad, San Diego, CA). One-way ANOVA was used among groups, followed by the Mann–Whitney U test for post hoc comparisons to determine the P values. The statistical significance of differences in the survival of mice from the different groups was determined by the log-rank test using the same program.

Chemistry
The purity of Jac-A was verified with NMR and HPLC, and the purity of Jac-A was 97%. *Jacarehyperol A* [17], isolated from *Hypericum japonicum* Thunb.ex Murray, yellow powder; $^1$H-NMR (DMSO-d$_6$, 500 MHz, δ$_H$): 6.78 (1H, d, J = 10 Hz), 5.86 (1H, d, J = 10 Hz), 7.63 (1H, d, J = 8 Hz), 7.03 (1H, d, J = 8 Hz), 7.51 (1H, d, J = 8 Hz), 6.92 (1H, d, J = 8 Hz), 6.29 (1H, s); $^{13}$C-NMR (DMSO-d$_6$, 125 MHz, δ$_C$): 70.2, 71.0, 78.4 (×2), 79.2, 97.6, 98.0 (×2), 100.7 (×2), 101.4, 102.6, 102.9, 112.8, 113.0, 113.7 (×4), 115.7, 116.8, 128.2 (×2), 131.6, 132.7, 145.4, 145.9, 150.6, 151.0 (×2), 151.9, 159.8, 162.3, 162.8, 179.7 (×2), 179.9; ESIMS: m/z 667 [M – H$^-$] ; (−)-Gossypol (98% purity) was purchased from Sigma–Aldrich (Shanghai, CA).

Results

Screening active compounds
Fluorescence polarization was used to screen for Bcl-2 protein inhibitors. Jac-A was chosen as the candidate compound for further research because of its high affinity to Bcl-2 proteins. As shown in Figure 1 and Table 1, Jac-A can dose-dependently bind to Bcl-x$_L$, Bcl-2, and Mcl-1 with a K$_i$ value of 0.46 μM, 0.43 μM, and 1.69 μM, respectively, which near to the activity of positive control (−)-Gossypol, a known Bcl-2 protein inhibitor.

Predicting the binding modes of Jac-A with Bcl-x$_L$
To map the binding site of Jac-A, we built complex structure of the compound with Bcl-x$_L$ by docking (Figure 2). Jac-A contains two xanthones and can generates many conformations by rotating the C10-O and O–C3” bonds. The best binding model is shown in Figure 2A, in which the two xanthones of Jac-A exhibit two different orientations with a 90° dihedral angle and occupy three sub-pockets (P2, P4, and P5). The three sub-pockets play an important role in binding with pro-death BH3-only proteins and ligands. The P2 pocket, formed by residues Tyr-101, Ala-104, Leu-108, Val-126, Asn-135, Ala-142, and Ser-145, makes hydrophobic contacts with the aromatic H ring of Jac-A. In the P4 pocket, the aromatic B ring, double bond of the A ring, and the two methyl groups at C-3 make hydrophobic contacts with the hydrophobic pocket formed by residues Glu-96, Phe-97, Gly-138, and Tyr-195. Moreover, the hydrophobic group D ring and isopropyl at C-2” of Jac-A can generate a hydrophobic interaction with the P5 pocket formed by residues Leu-130, Arg-132, and Arg-139. In addition, the three

Figure 1 The inhibitory activity of Jac A to Bcl-2 proteins. (A) The structure of Jac A. (B) The inhibitory curves of Jac-A to Bcl-x$_L$, Bcl-2, and Mcl-1 by fluorescence polarization. The substrate for the assay was a 5-FAM-labeled Bid BH3 domain peptide (amino sequence: EDIIRIARHLAQVGDSDMR).
hydroxyl groups of Jac-A are predicted to form a hydrogen bond with the polar atoms of residues Gly-138, Tyr-101, and Glu-129 (Figure 2B).

Anti-cancer activity of Jac-A
Bcl-xL, Bcl-2, and Mcl-1 are overexpressed in multiple cancer cells and contribute to tumour drug resistance [18]. Since Jac-A binds to Bcl-xL, Bcl-2, and Mcl-1 with high affinity and inhibits their interactions with the BH3 domain of proapoptotic proteins, we elected to study the effect of Jac-A on cancer cells. Using the MTT assay, we tested the cytotoxicity of Jac-A against various human cancer cell lines. Remarkably, Jac-A induced a dose-dependent reduction in cell viability compared to positive control doxorubicin (Table 2). Jac-A exhibited cytotoxic potency against breast cancer cells (MBA-MB-231, T47D), colon cancer cells (LOVO), lung cancer cells (A549), liver cancer cells (HepG2), and leukaemia cancer cells (K562, HL-60, THP-1). Jac-A showed strongest activity against leukaemia cells with IC_{50} values from 6.52 to 9.92 μM. Our results demonstrate that Jac-A possesses broad anti-cancer effects.

In the next experiment, we tried to elucidate whether the cytotoxicity caused by Jac-A is from apoptosis. K562 cells were treated with different concentrations of Jac-A and the cytotoxic effects were evaluated by Annexin V and PI dual staining [19]. Annexin V/PI staining in the control group showed a large viable cell population (marked as PI−AV−) and a small amount of early apoptotic (PI−AV+), late apoptotic (PI+AV+), and dead cells (PI+AV−). Jac-A resulted in a shift from viable cells to early and late apoptotic cell population with little change in the dead cell population, especially at the concentration of 10 μM. As shown in Figure 3, flow cytometry analysis showed that Jac-A induced K562 cell apoptosis in a dose-dependent manner. Approximately 2.3% ± 0.9%, 9.5% ± 1.2%, 14.4% ± 2.3%, and 44.7% ± 3.3% PI + AV+ cell populations and 1.1% ± 0.7%, 10.2% ± 1.4%, 23.5% ± 3.1%, and 22.1% ± 2.3% PI-AV+ cell populations were detected in the 0.1, 1, 5, and 10 μM Jac-A groups, respectively. While no significant apoptotic population was detected in the control group or DMSO group.

Table 1 Binding affinity of Jac-A and (∓)-gossypol with Bcl-xL, Bcl-2 or Mcl-1 determined by a fluorescence polarisation assay (Ki, μM)

| Compound      | Bcl-xL | Bcl-2 | Mcl-1 |
|---------------|--------|-------|-------|
| Jac-A         | 0.46   | 0.43  | 1.69  |
| (∓)-Gossypol  | 1.01   | 0.95  | 0.86  |

Figure 2 The predicted binding mode of Jac-A in the binding site of Bcl-xL (PDB code 2YXJ). (A) The binding site and orientation of Jac-A in the hydrophobic groove of Bcl-xL. Jac-A occupies three key sub-pockets (P2, P4, and P5) which play an important role in binding with pro-death BH3-only proteins and ligands. The protein is rendered in green, while the compound is rendered in purple. The P2, P4, and P5 binding pockets are labelled with dash circles. (B) The interaction model between Jac-A with Bcl-xL. Jac-A can form three hydrogen bonds with the polar atoms of residues Gly-138, Tyr-101, and Glu-129. Bcl-xL is rendered as a cartoon, while the residues in contact with the ligand and compound are rendered as sticks. The residues are rendered in green, and the compound is coloured in purple. The hydrogen bonds are represented by dotted black lines.
There was a significance difference in the amount of apoptosis cells in the Jac-A-treated groups compared with the control group. At the same conditions, the positive control (−)-Gossypol showed similar activity of induction apoptosis for K562 cells (Additional file 1: Figure S1). Moreover, Jac-A presented similar activity of apoptosis induction for other leukemia cells HL-60, THP-1 and colon cancer cells LOVO (Additional file 1: Figure S2-S4). After treated with 10 μM of Jac-A, 33.7% ± 3.1% PI + AV + and 29.2% ± 1.4% PI-AV + cell populations were detected in HL-60 cells, and 35.1% ± 2.4% PI + AV + and 27.8% ± 2.1% PI-AV + cell populations were detected in THP-1 cells. However, the activity of apoptosis induction for LOVO cells weaker than for leukaemia cells. Only 17.2% ± 1.6% PI + AV + and 7.89% ± 2.2% PI + AV + cell populations were detected in LOVO cells.

Jac-A activates caspases cascades

In the intrinsic apoptotic pathway, Bax and Bak, which are regulated by anti-apoptotic Bcl-2 proteins and BH3-only proteins, stimulate the release of cytochrome c from the mitochondrial intermembrane space into the cytosol. Then, cytochrome c induces apoptosis formation, with the activation of caspase-9 as the apical caspase [20]. Caspase-9 further activates the effector caspase-3, which cleaves several hundred cellular proteins, resulting in the

Table 2 The inhibitory activity of Jac-A on tumour cells via MTT Assay (IC50, μM, n = 4, mean ± SD)

| Compound | Breast cancer | Colon cancer | Lung cancer | Liver cancer | Leukaemia |
|----------|---------------|--------------|-------------|--------------|-----------|
|          | MBA-MB-231    | T47D         | LOVO        | A549         | HepG2     | K562      | HL-60     | THP-1     |
| Jac-A    | 22.61 ± 1.2   | 12.53 ± 1.6  | 33.24 ± 2.1 | 18.8 ± 1.6   | 10.71 ± 1.4 | 6.52 ± 0.36 | 9.01 ± 0.47 | 9.92 ± 0.95 |
| Doxorubicin | 0.13 ± 0.01  | 0.25 ± 0.02  | 0.24 ± 0.01 | 0.11 ± 0.01  | 0.34 ± 0.02 | 0.55 ± 0.03 | 0.68 ± 0.02 | 0.52 ± 0.03 |

*Positive control.

Figure 3 Jac-A caused K562 cell apoptosis. 10⁶ of K562 cells were treated with different concentrations of Jac-A for 48 h. Cells were then stained with Annexin V-FITC and propidium iodide, and analysed using flow cytometry. Four different cell populations marked as the following: live cell population (PI−AV−), early apoptosis (PI−AV+), late apoptosis (PI+AV+) and dead cells (PI+AV−). Cells were treated with no Jac-A (A), 0.5% DMSO (B), or 0.1, 1, 5, 10 μM Jac-A (C, D, E and F). The results are one representative of three independent experiments.
characteristic biochemical and morphological features associated with apoptosis, including chromatin condensation, nuclear fragmentation, and externalisation of phosphatidylserine [21]. To elucidate whether cytochrome c fits in Jac-A-induced apoptosis, Jac-A treated K562 cells were lysed, the cytosol and the mitochondrial membrane portion of the treated cells were obtained through a serial of ultra-centrifugation to probe cytochrome c in both portions using Western blot analysis. As shown in Figure 4A, release of cytochrome c into cytosol was detected in a dose-dependent manner from low as 3 μM of Jac-A at 48 h of treatment. Moreover, we checked the cleavage of caspase-9, caspase-3, and PARP by immunoblotting. As shown in Figure 4B, cleavages of caspase-9, caspase-3, and PARP were detected after K562 cells were treated with Jac-A for 48 h, which is consistent with the previous observation that some inhibitors of antiapoptosis proteins can induce the activation of caspases [22,23]. In addition, to investigate whether caspases play roles in Jac-A-induced apoptosis of K562 cells, we did another apoptosis assay to examine the effects of a broad-spectrum caspase inhibitor,
Z-VAD-FMK on Jac-A-induced apoptosis in K562 cells. K562 cells were first treated with or without different concentrations of Z-VAD-fmk for 4 h, followed by the treatment of Jac-A (10 μM) for 48 h. Cells undergoing apoptosis were measured using by phosphatidylserine (PS) externalization using Annexin V/FITC in the presence of propidium iodide (PI). As exhibited in Figure 4C, Jac-A-induced apoptosis was inhibited in a concentration dependent manner by the Z-VAD-FMK. These findings suggest that Jac-A-induced apoptosis in K562 cells is partly caspase-dependent.

Inhibition of the heterodimerization of antiapoptotic proteins with pro-apoptotic proteins

To confirm that Jac-A binds to anti-apoptotic Bcl-2 family members and competes with binding of pro-apoptotic proteins, co-immunoprecipitation was performed to analyse if these interactions are disrupted by Jac-A. As illustrated in Figure 5, 6 μM of Jac-A treatment started to clearly inhibit the binding of Bcl-xL and Bax. Exposed to 12 μM of Jac-A treatment, little Bcl-xL was seen to bind to Bax. Similarly, less Bcl-2 was observed to bind to Bax at 12 μM of Jac-A treatment than other doses of Jac-A and vehicle control treatment. Moreover, Jac-A also inhibited the binding of Mcl-1 to Bak but the inhibitory effect was presented at 12 μM of Jac-A treatment. These observations demonstrate that Jac-A-induced apoptosis of K562 cells is involved in inhibiting the heterodimerization of antiapoptotic proteins (Bcl-xL, Bcl-2, and Mcl-1) with pro-apoptotic proteins (Bax, Bak).

Inhibitory effect of Jac-A on K562 cell growth in xenograft mice

To test if Jac-A can inhibit K562 tumour cell growth in vivo, we used nude mice that were injected subcutaneously with K562 cells into the right flank. The K562-bearing mice were randomly divided into four groups (ten mice in each group) and treated with vehicle and Jac-A at a dose of 2, 10, or 50 mg/kg daily for 21 days. As shown in Figure 6A–C, Jac-A dose-dependently inhibited tumor growth. Higher doses of Jac-A had a better inhibitory effect and longer observed survival time (Figure 6D). Interestingly, the body weight of the control group and mice treated with the low-dose of Jac-A were significantly lower than mice from higher dose groups (2, 10, or 50 mg/kg) (Figure 6E). This phenomenon can be attributed to the low toxicity and therapeutic effect of Jac-A. In the process of this experiment, the tumors' growths of mice from higher dose groups were significantly inhibited and the quality of life was better than control group and low-dose group. The absorbed energy from food mainly used for keeping the growth of tumor for mice in control group and low-dose group, which contribute to losing weight. However, this phenomenon improved in higher dose groups. This finding suggests that Jac-A can effectively inhibit the growth of tumor in vivo with low toxicity.

Discussion

Many anti-cancer drugs have significant side effects, and some cancers are drug resistant [24-26]. Therefore, potential anti-cancer compounds are needed in pharmaceutical development. Natural products, with inherently larger-scale structural diversity than synthetic compounds, are the major resources of bioactive agents and will continue to provide the most candidates for new drug discovery. Many natural product resources have been used as medicine, such as those in traditional Chinese medicine. Active compounds from medicinal plants are generally biologically friendly, because of their clinical use. Here, we identified a new natural Bcl-2 inhibitor Jac-A with potential therapeutic use in murine models of human leukaemia via high throughput screening of our in-house NPL and biological testing.

Jac-A, a characteristic constituent of H. japonicum, was firstly reported by Kyoko Ishiguro et al. and characterized by its inhibitory effect on PAF-induced hypotension [17]. In this work, Jac-A was identified as a new inhibitor of Bcl-2 proteins. We found that Jac-A can compete for binding to BH3 domain of Bcl-2.
proteins with proapoptosis proteins in the FP-based binding experiments. This result was confirmed by the co-immunoprecipitation experiment whose results showed Jac-A can inhibit the heterodimerization between antiapoptotic proteins (Bcl-xL, Bcl-2, and Mcl-1) with pro-apoptotic proteins (Bax and Bak) in K562 cells. Moreover, Jac-A showed potent activity in inducing the apoptosis of K562 cells. Simultaneously, we found that Jac-A can promote the release of cytochrome c into cytosol and trigger the activation of downstream protein containing caspase-9, caspase-3, and PARP. Additionally, we confirmed that Jac-A-induced apoptosis in K562 cells is partly caspase-dependent based on a broad-spectrum caspase inhibitor Z-VAD-FMK. In the in vivo test, Jac-A also showed a dose-dependently inhibition for human K562 cell growth in xenograph tumor mice with low toxicity.

Conclusions

In summary, we identified the Jac-A, a characteristic component of *H. japonicum*, induces apoptosis in K562 cells by inhibiting the heterodimerization of Bcl-xL/Bcl-2 with Bak, and Mcl-1 with Bax. Together with anticancer activity in vivo, these works not only discovered a new lead compound for cancer therapy with low toxicity, but also provided evidence for using *H. japonicum* as an anticancer herb.
Additional file

Additional file 1: Figure S1. (--)Gossypol caused K562 cells apoptosis. 10^6 of K562 cells treated with 5 and 10 μM of (--)Gossypol for 48 h Cells were then stained with Annexin-V-FITC and propidium iodide, and analysed using flow cytometry. Cells were treated with no Jac-A (A), 0.5% DMSO (B), or 10 μM Jac-A (C). The results are one representative of three independent experiments. Figure S2. Jac-A caused HL-60 cells apoptosis. 10^6 of HL-60 cells treated with 10 μM of Jac-A for 48 h. Cells were then stained with Annexin-V-FITC and propidium iodide, and analysed using flow cytometry. Cells were treated with no Jac-A (A), 0.5% DMSO (B), or 10 μM Jac-A (C). The results are one representative of three independent experiments. Figure S3. Jac-A caused THP-1 cells apoptosis. 10^6 of THP-1 cells treated with 10 μM of Jac-A for 48 h. Cells were then stained with Annexin-V-FITC and propidium iodide, and analysed using flow cytometry. Cells were treated with no Jac-A (A), 0.5% DMSO (B), or 10 μM Jac-A (C). The results are one representative of three independent experiments. Figure S4. Jac-A caused LOVO cells apoptosis. 10^6 of LOVO cells treated with 10 μM of Jac-A for 48 h. Cells were then stained with Annexin-V-FITC and propidium iodide, and analysed using flow cytometry. Cells were treated with no Jac-A (A), 0.5% DMSO (B), or 10 μM Jac-A (C). The results are one representative of three independent experiments.

Abbreviations
Jac-A: Jcacerythromycin A; Bcl-2: B-cell lymphoma 2; NPD: Natural products database; FP: Fluorescence polarisation; MTT: (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; TCM: Traditional Chinese medicine; H. japonicum: Hypericum japonicum Thunb.ex Murray; PAF: Platelet activating factor.

Competing interests
The authors declare that they have no competing interests.

Authors’ contributions
Designed the experiments: SDZ, HW and WDZ; Performed the experiments and analysed data: JY, XL, JGZ, RCY and YYD; Wrote the manuscript: SDZ; Edited the manuscript: H. upon ethic approval. This work was supported by the NCET-12-0070, NSFC (81230090), partially supported by Fundamental assistance in editing the manuscript. This work was supported by the NCET-12-0070, NSFC (81230090), partially supported by Fundamental assistance in editing the manuscript. This work was supported by the NCET-12-0070, NSFC (81230090), partially supported by Fundamental assistance in editing the manuscript. This work was supported by the NCET-12-0070, NSFC (81230090), partially supported by Fundamental assistance in editing the manuscript.

Acknowledgements
The authors thank Dr. Hongbin Wang and Dr. Jiang Li for kind assistance in editing the manuscript. This work was supported by the NCET Foundation, NSFC (81102016), National Basic Research Program (973 Program) and Shanghai Engineering Research Center for Functional and Bioactive Natural Products (10DZ225100) and the Science and Technology Commission of Shanghai City (09DZ1975700). All authors read and approved the final manuscript.

Received: 14 March 2014 Accepted: 17 September 2014 Published: 22 September 2014

References
1. Wang XW, Mao Y, Wang NL, Yao XS: A new phloroglucinol diglycoside derivative from Hypericum japonicum Thunb. Molecules 2008, 13(11):2796–2803.
2. Wang N, Li P, Wang Y, Peng W, Wu Z, Tan S, Liang S, Shen X, Su W: Hepatoprotective effect of Hypericum japonicum extract and its fractions. J Ethnopharmacol 2008, 116(1):1–6.
3. Zuo GY, An J, Han J, Zhang YL, Wang GC, Hao XY, Bian ZQ: Isocaracilein from the Chinese herb Hypericum japonicum: potent antibacterial and synergistic effects on clinical methicillin-resistant staphylococcus aureus (MRSA). Int J Mol Sci 2012, 13(10):8210–8218.
4. Gross A, McDonnell JM, Korsmeyer SJ: BCL-2 family members and the mitochondria in apoptosis. genes Dev 1999, 13(15):1889–1911.
5. Eneydi I, Ling Y, NaPo K, Toronyi T, Wu Y, Cao Y, Guo R, Li B, Kim H, Huang Y, Long YQ, Roller PP, Yang D, Wang S: Discovery of small molecule inhibitors of Bcl-2 through structure-based computer screening. J Med Chem 2001, 44(25):9313–9324.
6. Adams JM, Cory S: The BCL-2 apoptotic switch. Cell Death and Differentiation. Oncogene 2007, 26(10):1324–1331.
7. Amundson SA, Myers TG, Suddeter D, Kitad S, Reed JC, Tomace AJ Jr: An informatics approach identifying markers of chemosensitivity in human cancer cell lines. Cancer Res 2005, 65(14):6074–6110.
8. Vogler M, Weber K, Dondelinger F, Schulze-Osthoff K, Dyer MJ, Cohen GM: Different forms of cell death induced by putative BCL2 inhibitors. Cell Death Differ 2009, 16(7):1084–1099.
9. Feng Y, Ding X, Chen T, Chen L, Li J, Lu X, Shen X, Chen K, Jiang H, Wang H, Liu X, Luedde T, Witzig T, Li JS, Li JS, Li JS: Synergistic effects on clinical methicillin-resistant staphylococcus aureus (MRSA). Int J Mol Sci 2012, 13(10):2734–2753.
10. Satter M, Liang H, Welsh D, Meadows RP, Harlan JE, Eberstadt M, Yoon Hyon-Young, Wu CB, Buss MN, Alain Thompson CB, Fesik SW: Structure of a Bak peptide complex: recognition between regulators and its inhibitors of BCL-xL. Science 1997, 275(5302):983–986.
11. Tallman MS, Gilliland DG, Rowe JM: Drug therapy for acute myeloid leukemia. Blood 2005, 106(4):1154–1163.
12. Maltseva-Coleska Z, Wang R, Fang X, Pan H, Toronyi T, Yu LP, Roller PP, Kowal K, Saito NG, Stuckey IA, Wang S: Development and optimization of a binding assay for the XIAP BIR3 domain using fluorescence polarization. Anal Biochem 2004, 332(2):261–273.
13. Kluck RM, Bossy-Wetzel E, Green DR, Newmeyer DO: The release of cytochrome c from mitochondria: a primary site for Bcl-2 regulation of apoptosis. Science 1997, 275(5303):1132–1136.
14. Zhang MC, Liu HP, Tian ZK, Griffith BN, Ji M, Li QQ: Gossypol induces apoptosis in human PC-3 prostate cancer cells by modulating caspase-dependent and caspase-independent cell death pathways. Life Sciences 2007, 80(8):767–774.
15. Snowden RT, Sun XM, Dyer MJS, Cohen GM: Bisindolylmaleimide IX is a potent inducer of apoptosis in chronic lymphocytic leukaemic cells and activates cleavage of Mcl-1. Leukemia 2003, 17(10):1981–1989.
16. Dewson G, Snowden RT, Almond JB, Dyer MJS, Cohen GM: Conformational change and mitochondrial translocation of Bax accompany proteasome inhibitor-induced apoptosis of chronic lymphocytic leukemia cells. Oncogene 2003, 22(17):2643–2654.
17. Ishiguro K, Nagata S, Oka H, Yamaki M: Bisanthrones from Hypericum japonicum: inhibitors of PAF-induced hypotension. Planta Med 2002, 68(5):258–261.
18. Buclarminki JW: Novel anticaner drug discovery. Curr Opin Chem Biol 1999, 3(4):500–509.
19. Mao S, Shi X, Zhang H, Wang S, Sun J, Hua W, Mao Q, Zhao Y, Zhang C: Proliferation-attenuating and apoptosis-inducing effects of trypaflavin on human chronic myeloid leukemia k562 cell line in vitro. Int J Mol Sci 2011, 12(8):3831–3845.
20. Green DR, Kroemer G: The pathophysiology of mitochondrial cell death. Science 2004, 305(5684):626–629.
21. Cohen GM: Caspases: the executioners of apoptosis. Biochem J 1997, 326(Pt 1):1–16.
22. Trudel S, Li ZH, Rauw J, Tiedemann RE, Wen XY, Stewart AK: Preclinical studies of the pan-Bcl inhibitor obatoclax (GX15-070) in multiple myeloma. Blood 2007, 109(12):4530–4538.
23. Mohammad RM, Goustin AS, Aboukameel A, Chen B, Banerjee S, Wang G, Nikolovska-Coleska Z, Wang S, Al-Katib A: Preclinical studies of TW-37, a new nonpeptidic small-molecule inhibitor of Bcl-2, in diffuse large cell lymphoma xenograft model reveal drug action on both Bcl-2 and Mcl-1. Clin Cancer Res 2007, 13(7):2226–2235.
24. Frame D: New strategies in controlling drug resistance in chronic myeloid leukemia. Am J Health Syst Pharm 2007, 64(24 Suppl 15):S16–S21.
25. Gorre ME, Mohammed M, Ellwood K, Hsu N, Paquette R, Rao PN, Sawyers CL: Clinical resistance to STI-571 cancer therapy caused by BCR-ABL gene mutation or amplification. Science 2001, 293(5531):876–880.
26. Hochhaus A, Kreil S, Corbin AS, La Rosee P, Muller MC, Lahaye T, Hanfstein B, Schoch C, Cross NC, Berger U, Gschaidmeier H, Druker BJ, Hehlmann R: Molecular and chromosomal mechanisms of resistance to imatinib (STI571) therapy. Leukemia 2002, 16(11):2190–2196.

doi:10.1186/1471-2407-14-689
Cite this article as: Zhang et al.: Jacarehyperol A induced apoptosis in leukaemia cancer cell through inhibition the activity of Bcl-2 proteins. BMC Cancer 2014 14:689.