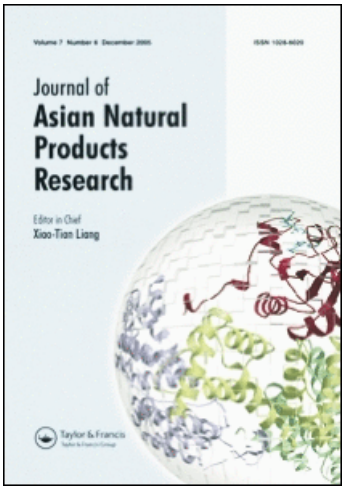


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[a] State Key Laboratory of Natural and Biomimetic Drugs, Peking University, Beijing, China [b] School of Traditional Chinese Materia Medica, Shenyang Pharmaceutical University, Shenyang, China

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# Pro-apoptotic and microtubule-disassembly effects of ardisiacrispin (A + B), triterpenoid saponins from *Ardisia crenata* on human hepatoma Bel-7402 cells

Min Li[a], Shao-Yin Wei[a], Bo Xu[a], Wei Guo[a], Dai-Lin Liu[b], Jing-Rong Cui[a]* and Xin-Sheng Yao[b]

[a]*State Key Laboratory of Natural and Biomimetic Drugs, Peking University, Beijing 100083, China;* [b]*School of Traditional Chinese Materia Medica, Shenyang Pharmaceutical University, Shenyang 110016, China*

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Ardisiacrispin (**A** + **B**) is a mixture of ardisiacrispins **A** and **B**, derived from *Ardisia crenata* with a fixed proportion (2:1). The present study was conducted to investigate its anticancer activity on human cancer cells and its underlying mechanism of action. The ($IC_{50}$)s of ardisiacrispin (**A** + **B**) on proliferation of several human cancer cell lines were in the range of 0.9–6.5 μg/ml by sulphorhodamine B-based colorimetric assay, in which Bel-7402 was the most sensitive cell line. Moreover, ardisiacrispin (**A** + **B**) induced dose-dependent apoptosis in Bel-7402 cells at doses of 1–10 μg/ml by flow cytometry, and resulted in the changes of the mitochondrial membrane depolarization, membrane permeability enhancement, and nuclear condensation in a dose-dependent manner through high-content screening analysis. Furthermore, ardisiacrispin (**A** + **B**) could disassemble microtubule in Bel-7402 cells; the fluorescence intensity of microtubules decreased at the concentration of 5–20 μg/ml. These findings suggest that ardisiacrispin (**A** + **B**) could inhibit the proliferation of Bel-7402 cells by inducing apoptosis and disassembling microtubule.

**Keywords:** *Ardisia crenata*; ardisiacrispin; apoptosis; microtubule; anticancer



## 1. Introduction

*Ardisia crenata* Sims, one of the species of genus *Ardisia*, has been used to treat several kinds of diseases including tonsillitis, toothache, trauma, arthralgia, and so on, in folk medicine for a long time in Guizhou province of China [1]. Some triterpenoid saponins in *A. crenata* exhibited cytotoxic effect on tumor cells, immunomodulatory and antiviral activities [2–4]. Ardisiacrispins **A** and **B**, two triterpenoid saponins of *Ardisia crispa* origin, gave contractive responses of the isolated rat uterus [5]. Importantly, ardisiacrispins **A** and **B** were common triterpenoid saponins with the similar biological properties in *A. crenata* [6], *A. crispa* [5], and New Zealand *Myrsine* species [3]. Moreover, ardisiacrispins **A** and **B** from New Zealand *Myrsine* species exhibited their cytotoxicities toward tumor cell lines of mice, but it is unclear whether ardisiacrispins **A** and **B** from *A. crenata* possess anticancer property to human cancer cell lines.

To study the anticancer activity of the mixture of ardisiacrispins **A** and **B**, named ardisiacrispin (**A** + **B**) with a fixed proportion (2:1) that was similar to the yield of these two compounds in nature (Figure 1), we examined its *in vitro* cytotoxicity in a panel of human cancer cell lines and investigated its underlying mechanism of action toward Bel-7402 cells.

*Corresponding author. Tel.: +86-10-82802467. Fax: + 86-10-82802467. Email: jrcui@bjmu.edu.cn

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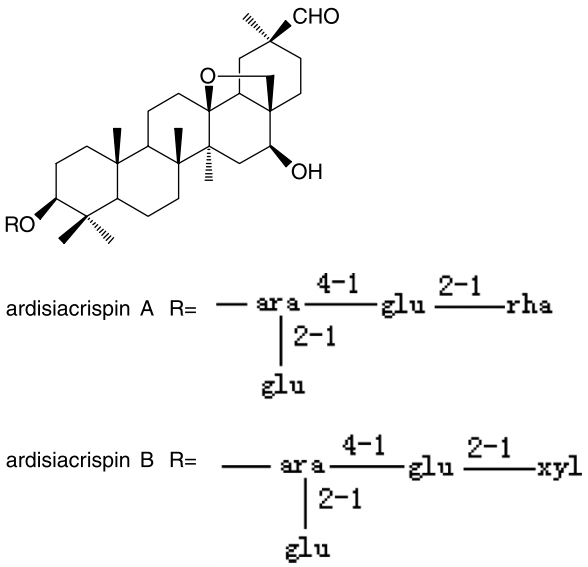


Figure 1. Chemical structures of ardisiacrispin **A** and **B**.



## 2. Results

### *2.1 Cytotoxicity of ardisiacrispin (A + B) on cancer cell lines*

After 48-h exposure, the cytotoxicity of ardisiacrispin (**A** + **B**) on several cancer cell lines was evaluated using sulphorhodamine B (SRB) assay. Ardisiacrispin (**A** + **B**) showed different cytotoxicities with respect to the cell lines, and the half-inhibitory concentrations ($IC_{50}$) of the cell viability were determined as 0.9, 2.0, 2.3, 5.0, 6.2, and 6.5 μg/ml for Bel-7402, KB, HeLa, SKOV-3, BGC-823, and MCF-7 cell lines, respectively (Table 1).

Table 1. Cytotoxic activity of ardisiacrispin (**A** + **B**).

| Cell lines | $IC_{50}$ (μg/ml) |
|---|---|
| Bel-7402 | 0.9 |
| KB | 2.0 |
| HeLa | 2.3 |
| SKOV-3 | 5.0 |
| BGC-823 | 6.2 |
| MCF-7 | 6.5 |

$IC_{50}$ values were determined by SRB assay on different human cancer cell lines for 48 h.

### *2.2 Pro-apoptotic effect of ardisiacrispin (A + B) on Bel-7402 cells*

To assess whether ardisiacrispin (**A** + **B**) induce apoptosis, we first checked the appearance of sub-$G_1$ peak, a specific fraction for apoptosis in cell cycle distribution by flow cytometry analysis. As illustrated in Figure 2, there were prominent apoptosis rate for 7.5 and 10.0 μg/ml ardisiacrispin (**A** + **B**) with a 24-h incubation.

To confirm the pro-apoptotic effect of ardisiacrispin (**A** + **B**) in Bel-7402 cells, we detected alterations in mitochondrial mass, as well as changes in f-actin content and nuclear fluorescence intensity. As shown in Figure 3(A), the mitochondrial mass showed a dose-dependent increase, and the value for higher concentration of ardisiacrispin (**A** + **B**) was much more than that of vinblastine. An early increase in f-actin content detected by labeling with phalloidin, an f-actin-specific stain, has been reported as a potential parameter related to apoptotic changes [7]. The f-actin area showed a lightly dose-dependent increase (Figure 3(B)). Though all values of ardisiacrispin (**A** + **B**) at different

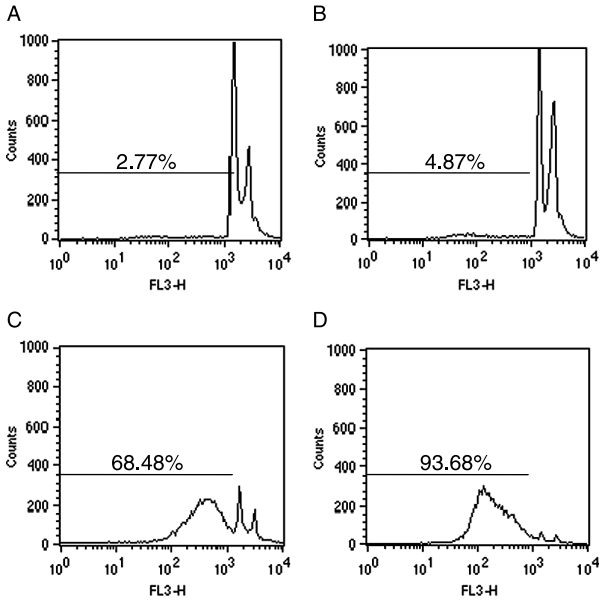


Figure 2. Pro-apoptotic effect of ardisiacrispin (**A** + **B**). Human hepatoma Bel-7402 cells were exposed to (A) solvent PBS, (B) 5.0 μg/ml, (C) 7.5 μg/ml, and (D) 10.0 μg/ml of ardisiacrispin (**A** + **B**) for 24 h. Apoptotic fraction was determined using flow cytometry.



concentrations were much lower than that of vinblastine, the nuclear fluorescence intensity also showed a dose-dependent increase (Figure 3(C)).

### *2.3 Effect of ardisiacrispin (A + B) on cellular microtubular cytoskeleton*

To determine whether ardisiacrispin (**A** + **B**) could affect microtubules, the effect of ardisiacrispin (**A** + **B**) on the microtubule cytoskeleton was investigated using fluorescence microscopy on Bel-7402 cells. As shown in Figure 4, the microtubule cytoskeleton appeared rich and intact in untreated cells (Figure 4(A)), whereas 0.1 μM colchicine, a well-known compound to target tubulin, resulted in cellular microtubule disassembly (Figure 4(B)). And ardisiacrispin (**A** + **B**) induced a dose-dependent disassembly of the microtubule network (Figure 4(C)–(F)), and 20 μg/ml ardisiacrispin (**A** + **B**) induced a complete disassembly.

Compartmental analysis is a multi-channel high-content screening (HCS) BioApplication that enables the simultaneous quantification of the presence of several different macromolecules in or between different regions or compartments in cells [8]. We defined the nuclear region as a primary object with fluorescent marker diamidino phenylindole (DAPI). Then, we defined two different regions of the cell, such as circle and circle spots by primary object. Quantification of the cellular microtubules with fluorescence analysis in a microplate showed that the average intensity of fluorescence staining gradually decreased when Bel-7402 cells were treated with increasing concentrations of ardisiacrispin (**A** + **B**) (Table 2).

## 3. Discussion

Several triterpenoid saponins with biological activities have been isolated from *A. crenata* such as ardisiacrispins **A**–**D** and ardisicrenosides **A**–**D** [5,6,9]. To assess whether ardisiacrispins **A** and **B** from *A. crenata* possess anticancer property to human cancer cell lines, we extracted ardisiacrispins **A** and **B** from *A. crenata*, mixed these two compounds

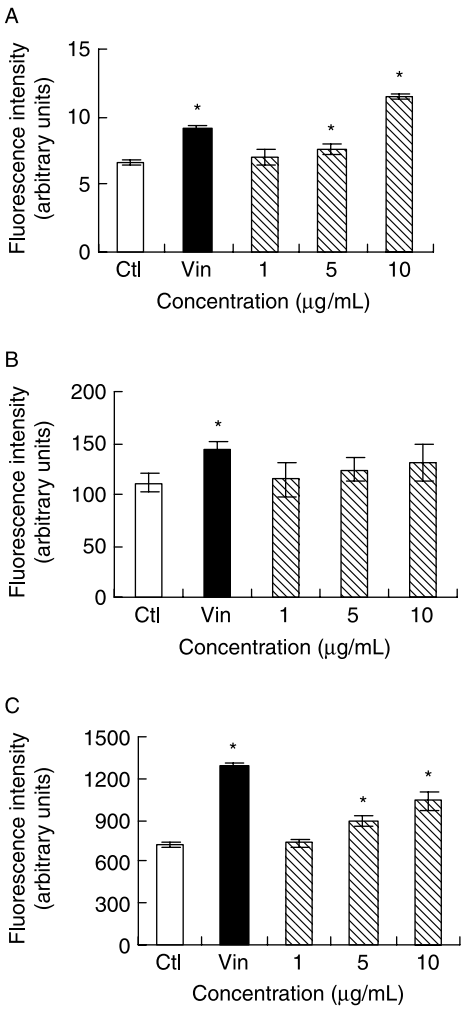


Figure 3. Apoptotic criteria of increasing concentrations of ardisiacrispin (**A** + **B**) on Bel-7402 cells for 24 h. (A) Mitochondrial masses were stained with MitoTracker Red. (B) F-actins were stained with Alexa Fluor 488 phalloidin. (C) Nuclei were stained with Hoechst 33342. The fluorescence intensities were quantified by HCS system (KSR). DMSO (0.1%) (Ctl) and 1 μmol/l vinblastine (Vin) were used as negative and positive controls, respectively. Results are presented as mean ± SD. $*P < 0.05$ *vs*. negative control. The analysis was measured in at least 100 cells in each well.

with a fixed proportion (2:1) that was similar to the yield ratio of the two compounds in nature, then investigated its anticancer activity on human cancer cells and its underlying mechanism of action. In the current study, ardisiacrispin (**A** + **B**) was shown to be potent inhibitors of tumor cell proliferation *in vitro*. Meanwhile, it was demonstrated that ardisiacrispin (**A** + **B**) induced apoptosis in a dose-dependent way. Furthermore, the antiproliferative action of the compound was linked to microtubule dysfunction.

A common feature of triterpenoid saponins from different origins is their cytotoxicity on cancer cell lines. For example, asterlingulatosides **C** and **D**, two analogs of ardisiacrispins **A** and **B**, were demonstrated their cytotoxic activity on human leukemia HL-60 cells with ($IC_{50}$) of 8.8 and 6.1 μmol/l, respectively [10]. Our work showed that ardisiacrispin (**A** + **B**) was potent toward human hepatoma Bel-7402 cells and several other cancer cells with ($IC_{50}$) ranged from 0.9 to 6.5 μg/ml. The relationship between structure and bioactivity, and different cell sensitivity remain to be elucidated.

Apoptosis is an important homeostatic mechanism that balances cell division and cell death. Induction of apoptosis in cancer cells is one strategy for anticancer drug development. Changes in mitochondria play a central role in apoptosis, and mitochondria releases apoptogenic factors through the outer membrane and dissipates the electrochemical gradient of the inner membrane, which is thought to occur via the formation of the mitochondria permeability transition [11]. Ardisiacrispin (**A** + **B**) gave a remarkable increase of mitochondrial mass, indicating the diminishing membrane potential of mitochondria. The actin cytoskeleton is central to many cell processes including membrane traffic, and its changes have been reported as a potential parameter related to apoptotic changes [12]. For example, thrombin appears to increase epithelial permeability by receptor-mediated enhancing of the appearance of actin network in airway epithelial cells [13]. Similarly, the fluorescence intensity of f-actin of the cells increased after the exposure to ardisiacrispin (**A** + **B**). Cells undergoing apoptosis generally exhibit one of two types of nuclear change, fragmentation,

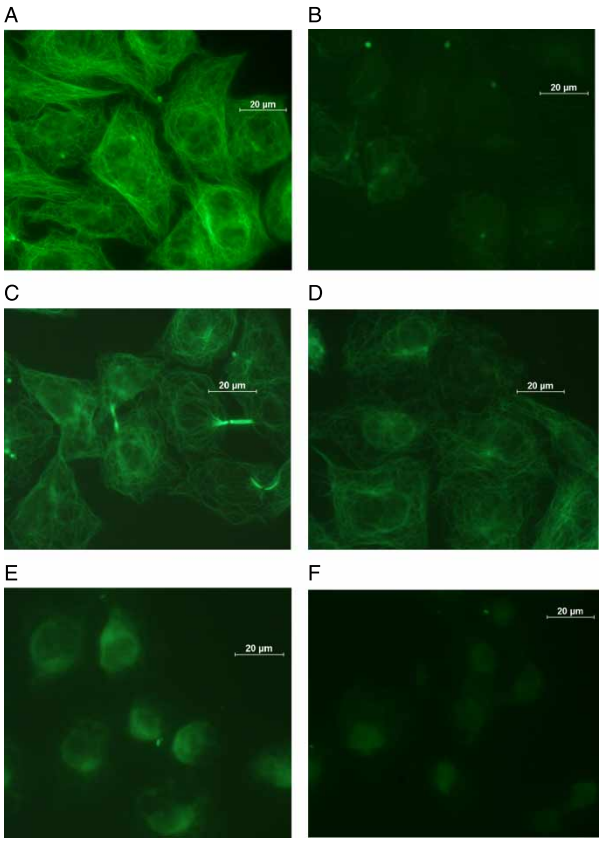


Figure 4. Demonstration of microtubule disassembly by immunofluorescence staining. Bel-7402 cells were treated for 6 h with (A) 0.1% DMSO, (B) 0.1 μM colchicine and (C, D, E, F) 5, 7.5, 10, and 20 μg/ml of ardisiacrispin (**A** + **B**). Microtubule network was labeled by α-tubulin staining with a specific antibody, followed by an incubation with a second antibody-FITC conjugate. Scale bar: 20 μm.

or condensation, and nuclear condensation appears to bind more fluorescent dye in smaller area so as to increase the fluorescence intensity. Hence, the changes of mitochondrial membrane potential, membrane permeability, as well as nuclear condensation were involved in the apoptosis induced by ardisiacrispin (**A** + **B**).

Table 2. Effect of ardisiacrispin (**A** + **B**) on microtubule disassembly measured using HCS assay.

| | | Parameter/fluorescence intensity | |
|---|---|---|---|
| Agent | Concentration (μg/ml) | Circle | Circle spots |
| Control | 0 | 82.63 ± 1.61 | 187.51 ± 2.74 |
| Colchicine | 0.04 | 29.23 ± 1.07* | 63.29 ± 1.31* |
| Ardisiacrispin (**A** + **B**) | 5 | 73.50 ± 2.46* | 155.98 ± 8.20* |
| | 10 | 54.03 ± 2.39* | 108.28 ± 7.90* |
| | 20 | 7.31 ± 3.18* | 12.67 ± 3.59* |

Bel-7402 cells were treated with different concentration of ardisiacrispin (**A** + **B**) for 6 h. DMSO (0.1%) and 0.04 μg/l colchicine were used as negative and positive controls, respectively. Results are presented as mean ± SD in triplicate. *$P < 0.05$ *vs*. control. Circle is a cellular region used to quantify the presence of a fluorescent macromolecule. Circle spots are any discrete objects that fall within the circle area.

Microtubules are essential for cell transport and cell division in all eukaryotes. Accumulated evidences have shown that microtubule dynamics may play a crucial role in the passage through the metaphase/anaphase checkpoint [14]. Mitotic block by drugs at concentrations that suppress microtubule dynamics or alter microtubule mass induces apoptosis [15,16]. It is interesting to note that some of cytotoxic compounds with the structure of triterpenoid saponin interact with microtubules. Our preliminary result showed that ardisiacrispin (**A** + **B**) can cause disassembly of the microtubule network in the early stage of the addition of the compound, providing a new mechanism of antiproliferative activity for triterpenoid saponin. The underlying mechanism of ardisiacrispin (**A** + **B**) on microtubule-disassembly effect and apoptosis requires further investigation for the development of the compound.

In summary, our study showed that ardisiacrispin (**A** + **B**) could inhibit the proliferation of human hepatoma cells (Bel-7402) via inducing apoptosis and disassembling microtubule. The finding that ardisiacrispin (**A** + **B**) has a remarked anticancer activity on Bel-7402 cells also opens interesting perspectives for further exploration of the triterpenoid saponins and compounds of *A. crenata* Sims origin as potential anticancer agents.

## 4. Materials and methods

### *4.1 Chemicals and antibodies*

Ardisiacrispin (**A** + **B**) was extracted in School of Traditional Chinese Materia Medica, Shenyang Pharmaceutical University, as described by Jansakul [5]. Colchicine, vinblastine, SRB, DAPI, the mouse monoclonal antibody against human α-tubulin, and the secondary antibody against mouse IgG labeled with FITC were purchased from Sigma-Aldrich (St Louis, MO, USA). Multiparameter Apoptosis 1 HitKit (K04-0001-1) was obtained from Cellomics (Pittsburgh, PA, USA).

### *4.2 Cell culture*

Human hepatoma cells (Bel-7402), nasopharyngeal carcinoma cells (KB), uterine cervix carcinoma cells (HeLa), ovarian carcinoma cells (SKOV-3), gastric carcinoma cells (BGC-823), and breast carcinoma cells (MCF-7) were obtained from the American Type Culture Collection. All the cell lines were cultured at 37°C in a humidified atmosphere of carbon dioxide–air (5:95). The culture medium for all cells consisted of RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, antibiotics (penicillin 100 U/ml, streptomycin 100 μg/ml), and 2 mmol/l L-glutamine (GIBCO, Grand Island, New York, USA).

### *4.3 Assays of cytotoxicity*

The effect of ardisiacrispin (**A** + **B**) on cell proliferation was determined using a standard SRB-based colorimetric assay [17]. Briefly, the different adherent cell lines were seeded onto 96-well microplates (Costar 3599) at $5 \times 10^4$ cells/ml (180 μl). After 18–24 h, exponentially growing cells were exposed to increasing concentration (0.01–100 μg/ml) of ardisiacrispin (**A** + **B**) for 48 h. 0.1% DMSO was used as solvent control. Then, the medium was discarded. After the fixation by 10% trichloroacetic acid for 1 h in 4°C, 100 μl 0.4% SRB in 1% acetic acid were added. After a 10-min incubation, the SRB solution was discarded. The plate was washed with 1% acetic acid, and the cells were air-dried. Then, 200 μl of 10 mmol/l Tris was added to dissolve the crystals. And the absorbance was measured at 540 nm using a microplate multi-detection reader (FLUOstar OPTIMA, BMG, Offenburg, Germany). Detection was followed by computerized data acquisition and processing. Absorbance values were expressed as a percentage of untreated controls and the cytotoxicity was expressed as $IC_{50}$. To observe the morphological changes of cells treated by the compound, the images of the cells were captured using a digital sight system (DS-U1, Nikon, Japan).

### 4.4 Flow cytometry

Bel-7402 cells were plated at a density of $2 \times 10^5$ cells/ml. On the following day, different concentrations of ardisiacrispin (**A** + **B**) and control were added. After 24 h, cells were harvested by centrifugation at 1000 *g*, washed in PBS and fixed overnight in 70% ethanol at −20°C. After they were added to 0.1 mg/ml RNase for 30 min at 37°C, PBS was used again to wash and resuspend the cells. When it was time to assay, the cells were stained with 0.5 mg/ml of propidium iodide. Pro-apoptotic effect (sub-$G_1$ population of the cells) was determined with analytical flow cytometry using a BD-LSR flow cytometer (Franklin Lakes, NJ, USA) with an excitation/emission of 488/525 nm. Experiments were performed in triplicate and gave similar results.

### 4.5 Apoptosis analysis by HCS assay

HCS is a tool for generating data on multiple parameters in single cells as well as in populations of cells [18], which was used to measure pro-apoptotic effect of compounds on Bel-7402 cells by KineticScan™ Reader (KSR, Cellomics). Following exposure to 1, 5, and 10 μg/ml ardisiacrispin (**A** + **B**) and control for 24 h, cell fixation and staining for imaging analysis were performed according to the manufacturer's instruction of Multiparameter Apoptosis 1 kit (HitKit K04-0001-01, Cellomics). The kit product provides the reagents and optimized protocol for quantifying three fundamental parameters related to the process of apoptosis: (i) nuclear morphology, measured by a fluorescent nuclear dye, Hoechst 33342, (ii) mitochondrial mass and/or membrane potential, based on the uptake of the fluorescent dye, MitoTracker® Red, into the mitochondria of cells, and (iii) changes in f-actin content, determined by fluorescent Alexa Fluor® 488-phalloidin staining, proportional to the amount of f-actin present in the cell. Images and data of the cells were automatically obtained using KSR. Appropriate filter sets for the detection of three fluorophores were used, and the different fluorescent signals were recorded in three different image collection channels of the Kinetic Scan HCS Reader. The Cell Health Profiling BioApplication was used to acquire and analyze the images. The analysis was measured in at least 100 cells in each well. The experiments were run in triplicate.

### 4.6 Indirect immunofluorescence staining

Indirect immunofluorescence of the microtubule network on coverslips was performed as described previously [19].

### 4.7 Analysis of microtubule assembly/disassembly by HCS

The intensity of the fluorescent microtubule network was quantified by the HCS assay. Briefly, Bel-7402 cells were seeded into 96-well microplates (Costar 3603) at $1 \times 10^5$ cells/ml (100 μl/well) and allowed to attach overnight. Cells were treated with increasing concentration of ardisiacrispin (**A** + **B**) (5, 10, and 20 μg/ml) for 6 h. Then, the cellular microtubule network was detected with a mouse monoclonal microtubule antibody against α-tubulin (1:1500) in PBS supplemented with 25% fetal bovine serum. Binding of the primary antibody was visualized subsequently with a secondary antibody (goat against mouse IgG, labeled with FITC, 1:60) in PBS supplemented with 40% fetal bovine serum. Nuclei were stained with DAPI (0.2 μg/ml). Images and data of the cellular microtubule were automatically obtained using KSR [20].

### 4.8 Statistics

The statistical analysis was performed using the software of SPSS 13.0, and one-way ANOVA was applied to study the relationship between different variables. $P$ value $<0.05$ was considered to be statistically significant.

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