Effect of Costunolide and Dehydrocostus Lactone on Cell Cycle, Apoptosis, and ABC Transporter Expression in Human Soft Tissue Sarcoma Cells

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Key words
- *Saussurea lappa*
- Asteraceae
- dehydrocostus lactone
- soft tissue sarcoma
- cell cycle analysis
- ABCB1/MDR1
- ABCG2/BCRP1

Abstract

Human soft tissue sarcomas represent a rare group of malignant tumours that frequently exhibit chemotherapeutic resistance and increased metastatic potential following unsuccessful treatment. In this study, we investigated the effects of costunolide and dehydrocostus lactone, which have been isolated from *Saussurea lappa* using activity-guided isolation, on three soft tissue sarcoma cell lines of various origins. The effects on cell proliferation, cell cycle distribution, apoptosis induction, and ABC transporter expression were analysed.

Both compounds inhibited cell viability dose- and time-dependently. IC_{50} values ranged from 6.2 µg/mL to 9.8 µg/mL. Cells treated with costunolide showed no changes in cell cycle, little in caspase 3/7 activity, and low levels of cleaved caspase-3 after 24 and 48 h. Dehydrocostus lactone caused a significant reduction of cells in the G1 phase and an increase of cells in the S and G2/M phase. Moreover, it led to enhanced caspase 3/7 activity, cleaved caspase-3, and cleaved PARP indicating apoptosis induction. In addition, the influence of costunolide and dehydrocostus lactone on the expression of ATP binding cassette transporters related to multidrug resistance (ABCB1/MDR1, ABCC1/MRP1, and ABCG2/BCRP1) was examined using real-time RT-PCR. The expressions of ABCB1/MDR1 and ABCG2/BCRP1 in liposarcoma and synovial sarcoma cells were significantly downregulated by dehydrocostus lactone.

Our data demonstrate for the first time that dehydrocostus lactone affects cell viability, cell cycle distribution and ABC transporter expression in soft tissue sarcoma cell lines. Furthermore, it led to caspase 3/7 activity as well as caspase-3 and PARP cleavage, which are indicators of apoptosis. Therefore, this compound may be a promising lead candidate for the development of therapeutic agents against drug-resistant tumours.

Abbreviations

ABC transporter:	ATP binding cassette transporter protein
STS:	soft tissue sarcomas
SW-872:	human liposarcoma cell line
SW-982:	human synovial sarcoma cell line
TE-671:	human rhabdomyosarcoma cell line

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Introduction

STS represent a rare group of malignant tumours of various histologies which frequently exhibit aggressive characteristics both locally and in the formation of distant metastases [1]. The most important prognostic factors are the histological subtype and grade [2, 3]. Frequently, STS exhibit chemotherapeutic resistance and increased metastatic potential. The efficacy of chemotherapy, which is currently one of the most effective ways to treat metastatic cancers, is limited [4–7]. Of the various mechanisms that are involved in conferring resistance, upregulation of drug efflux transmembrane ABC transporters, such as P-glycoprotein (ABCB1/MDR1), multidrug resistance protein 1 (ABCC1/MRP1), and ABCG2/BCRP1, has become a major obstacle to cancer chemotherapy and seriously affects the clinical outcome [8–11]. ABCB1 is the best known drug transporter and seems to be induced in response to chemotherapeutic treatment. ABCB1 expression was the highest in the largest and most aggressive tumours in a range of soft tissue sarcomas [12]. Inhibition of ABCG2 in tumour cells has been reported to increase the intracellular drug concen-

tration and the efficacy of chemotherapeutics, whereas inhibition of ABCG2 in the liver and intestine may attenuate its defence against environmental toxicants [13]. The ABCC1 pump confers resistance to doxorubicin, daunorubicin, vincristine, colchicines, and several other compounds and has a very similar profile to ABCB1 [14]. Plants and their constituents have always played a central role in the search for new anticancer drugs. To date, about 73% of all approved anticancer drugs are either products derived from natural sources or developed based on knowledge won from natural products [15]. In a systematic bioactivity-based screening of plants used in traditional Chinese medicine, seventy-six plants were obtained in China and tested for their growth-inhibitory activity against tumour cells. Twenty-three of 253 extracts showed high activity [16]. One of these plants was *Saussurea lappa* Clarke (Asteraceae) which is traditionally used to treat asthma, cancer, cholera, rheumatism, chronic inflammations of the lung, indigestion, nausea, and vomiting [17,18]. Besides other terpenoid compounds [19,20], sesquiterpene lactones, including costunolide and dehydrocostus lactone, are the major components of the roots and have been reported to exhibit various biological activities [18,21,22]. Based on an improved knowledge of tumour biology, agents with novel and specific mechanisms of action may add to existing therapies for drug-resistant tumours.

In the present study, we investigated the effects of costunolide and dehydrocostus lactone on cell proliferation, cell cycle, apoptosis, and the expression of ABC transporters of three human soft tissue sarcoma cell lines of various origins.

Material and Methods

Plant material and extraction

Roots of *Saussurea lappa* Clarke were acquired at the medicinal plant market in Kunming, China, in November of 2002 and identified by Prof. Dr. X.-J. Hao, Kunming Institute of Botany, Chinese Academy of Sciences, Kunming, China. A specimen copy (#053-01-01-0-00) is on deposit in the herbarium of the Institute for Plant Sciences, Karl-Franzens University Graz, Austria. Fifty-five g fresh powdered material was successively and exhaustively extracted with petroleum ether and MeOH by Soxhlet extraction and dried under reduced pressure at 40 °C. The yields were 1.7 g and 19.8 g, respectively.

Isolation procedure

Costunolide and dehydrocostus lactone were isolated from the petroleum ether extract using preparative HPLC. Two hundred mg extract was dissolved in acetonitrile (40 mg/mL) and subjected to a preparative HPLC consisting of a Varian Prep Star pump (model SD-1) and a Dynamax absorbance detector model UV-1. Fractionation was performed with a VDSpher 100 RP18 column (250 × 25 mm, 10 µm). The mobile phase consisted of A: water and B: acetonitrile, and the following gradient was used: 0–10 min, 84% B; 10–15 min, 84–100% B. Flow rate was 20 mL/min. The collected fractions were dried under reduced pressure. Both compounds were identified using a Varian Unitylnova 400 MHz (400 MHz for ^{1}H and 100 MHz for ^{13}C) spectrometer at 25 °C using TMS as the internal standard. Both were measured in pyridine-d_5 (Sigma-Aldrich). The yield of costunolide was 39.5 mg. A second purification using the same parameters yielded 37.4 mg dehydrocostus lactone.

Sample preparation

Extracts and isolated compounds were dissolved in DMSO and diluted with culture medium. The final DMSO concentration never exceeded 0.5% and did not affect the cells. Vehicle-treated cells served as a control.

Cell culture

SW-872, SW-982, and TE-671 cell lines were obtained from CLS. They were cultured in Dulbecco's modified Eagle's medium (DMEM-F12; GIBCO®, Invitrogen), containing 5% foetal bovine serum (FBS; GIBCO®, Invitrogen), 1% L-glutamine (GIBCO®, Invitrogen), 100 units/mL penicillin (GIBCO®, Invitrogen), 100 µg/mL streptomycin (GIBCO®, Invitrogen), and 0.25 µg amphotericin B (PAA Laboratory) at 37 °C in a humidified atmosphere of 5% CO_2. The medium was replaced every 3 days, and cell passages were continued upon reaching confluence. CCRF-CEM leukaemia cells (provided by the German Cancer Research Center, Heidelberg, Germany) were grown in RPMI 1640 medium (GIBCO®, Invitrogen), 1% L-glutamine, 10% FBS, and 1% penicillin/streptomycin.

Cell viability assay

The CellTiter 96® AQ_{ueous} Assay (Promega) is composed of solutions of a novel tetrazolium compound MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) and an electron coupling reagent PMS (phenazine methosulfate). The quantity of the formazan product as measured by the absorbance at 490 nm is directly proportional to the number of living cells in the culture [23]. 1×10^4 cells (100 µL) were seeded into 96-well microtitre plates (Brand), and the assay was performed following the manufacturers' instructions. The commercial chemotherapeutics cisplatin and doxorubicin were used as positive controls.

Cell cycle analysis

After incubation with the respective IC_{50} concentrations of costunolide or dehydrocostus lactone for 48 and 72 h, cells were harvested by trypsinisation. Ice-cold ethanol (70%) was used to fix 5×10^5 cells for 10 min at 4 °C. After washing with PBS, the cell pellet was resuspended in propidium iodid (PI) staining buffer (50 µL/mL PI, RNAse A; Beckman Coulter) and incubated for 15 min at 37 °C. Cell cycle distribution was analysed by a FACS-Calibur (BD Biosciences) using ModFit software. To investigate cell proliferation, the proliferation index (Pi) and S-phase cell fraction (SPF) were calculated. Pi is expressed as $Pi = (S + G_2/M) / (G_0/G_1 + S + G_2/M)$. SPF is expressed as $SPF = S / (G_0/G_1 + S + G_2/M)$.

Annexin V-FITC/PI apoptosis assay

The FITC annexin V apoptosis detection kit (BD Biosciences) was performed following the manufacturers' instructions. Apoptotic cells were identified by resuspending 1×10^5 cells in 100 µL annexin V binding buffer containing 5 µL annexin V-FITC and 5 µL PI for 15 min at room temperature. Flow cytometry analysis was performed with FACS Calibur (BD Biosciences). 10000 events were collected. Cells were identified in the side scatter and forward scatter with a linear scale. Fluorescence signals were shown with a logarithmic scale. Compensation was performed by single annexin and PI measurements and analysed by FCS3 express software (De Novo software). Untreated cells were used as a negative control.

Caspase-3 apoptosis assay

After incubation with the respective IC_{50} concentrations of costunolide or dehydrocostus lactone for 24 and 48 h, cells were harvested by trypsinisation, fixed with formaldehyde for 10 min at 37 °C (2×10^6 cells/mL) and permeabilised with methanol. The pellet was resuspended in incubation buffer (PBS : FBS 1 : 200) and stained with FITC-conjugated monoclonal active caspase-3 antibody (Cell Signaling Technology). Cells were analysed by flow cytometry (FACSCalibur; BD Biosciences) performed with FACS-Diva software. Histograms were created using FCS3 express software (De Novo software). Untreated cells were used as a negative control.

Caspase-Glo® 3/7 assay

10 000 cells/well (100 µL) were treated with the respective IC_{50} concentrations of the compounds for 6–72 h and analysed regarding caspase activation using the Caspase-Glo® 3/7 assay according to the manufacturer's protocol. Luminescence was measured 30 min after adding the Caspase-Glo® 3/7 reagent (Caspase-Glo® substrate and buffer).

Western blot analysis

For total protein analysis, cells were resuspended in lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 50 mM NaF, 1 mM EDTA, 10% NP-40, 1% Triton-X and protease inhibitors), incubated on ice for 10 min and centrifuged at 15 000 rpm for 15 min. Aliquots of protein extracts (20 µg) were separated on 12% SDS-PAGE and electroblotted onto a 0.45 µm Hybond ECL nitrocellulose membrane (Amersham Biosciences). The membrane was blocked with 3% milk blocking buffer for 1 h and incubated with the primary antibodies for 2 h at room temperature. As primary antibodies, mouse polyclonal PARP antibody (#9542; New England Biolabs), rabbit polyclonal ABCB1/MDR1 (# sc-13131; Santa Cruz Biotechnology), and rabbit polyclonal ABCG2 (H-70) (# sc-25821; Santa Cruz Biotechnology) were used. The blots were developed using horseradish peroxidase-conjugated secondary antibodies (Dako) at room temperature for 1 h and the SuperSignal® West Pico chemoluminescent substrate (Thermo Scientific), in accordance with the manufacturers' protocol.

Real-time RT-PCR

Real-time RT-PCR was performed to determine the relative expression of the ABC transporter genes ABCB1/MDR1, ABCC1/MRP1, and ABCG2/BCRP1. Total RNA was isolated from treated and untreated cells with RNeasy Mini Kit (Qiagen), following the manufacturer's recommended protocol. DNA was digested with 1 U DNase (Fermentas) per µg RNA. One µg RNA was reverse-transcribed using RevertAid cDNA synthesis kit (Fermentas). Real-time PCR reactions were performed in triplicates using the Platinum SYBR Green Super Mix with ROX (Invitrogen) on AB7900HT (Applied Biosystems, Invitrogen). The housekeeping genes glyceraldehyde 3-phosphate dehydrogenase (GAPDH), β-actin (ACTB), and hypoxanthine phosphoribosyltransferase (hprt-n) served as an internal control due to their stable expression in different tissues. The following primers were used: ABCB1/MDR1 (multidrug resistance protein): forward GAGGAAGACATGACCAGGTA and reverse CTGTCGCATTATAGCATGAA; ABCC1/MRP1: forward AGTGGAACCCCTCTCTGTTTAAG and reverse CCTGATACGTCTTGGTCTTCATC; ABCG2/BCRP1 (breast cancer resistance protein): forward ACCTGAAGGCATTTACTGAA and reverse TCTTTCCTTGCAGCTAAGAC; GAPDH: forward AAGGTCGGAGTCAACGGA and reverse ACCAGAGTTAAAAGCAGCCCT; hprt-n: forward ATGGGAGGCCATCACATT and reverse ATGTAATCCAGCAGGTCAGCAA and ACTB: forward CTGGAACGGTGAAGGTGACA and reverse AAGGGACTTCCTGTAACAATGCA. The expression levels were calculated based on the $2^{-\Delta\Delta CT}$ method [24].

Statistical analysis

All values are expressed as mean values ± SD. Student's unpaired t-test was used to evaluate differences between treated groups and their respective controls. The significance of dose or time responses was assessed by repeated measures analysis. Graphic data was prepared with Sigmaplot® (Systat Software, Inc.). IC_{50} values were determined using the four parameter logistic curve.

Results

In a pharmacological screening with CCRF-CEM leukaemia cells, a petroleum ether and a MeOH extract of *Saussurea lappa* roots were investigated by the MTS viability assay. While the petroleum ether extract exhibited strong growth reduction (0.3 ± 0.1% of control cells at 10 µg/mL), the MeOH extract had no effect on the proliferation and viability of these cells (data not shown). Activity-guided fractionation of the petroleum ether extract led to the isolation of costunolide and dehydrocostus lactone (**Fig. 1**) as main and active compounds. Their structures were elucidated using ^1H and ^{13}C NMR experiments and by comparison with published data [25, 26].

To investigate the influence on cell growth, STS cell lines were exposed to 0, 0.5, 1.0, 5.0, 10.0, 25.0, 50.0, and 100.0 µg/mL of each compound for 24, 48, and 72 h. After these incubation periods, cells were measured by the MTS assay (n = 12). **Fig. 2A** and **B** show the 48 h values of the time- and dose-dependent inhibition of the cell viability. IC_{50} values are listed in **Fig. 2C**. Averaged across all cell lines (mean and SD of all obtained IC_{50} values), costunolide affected growth behaviour after 48 h by 9.7 ± 0.1 µg/mL (≙ 41.7 ± 0.4 µM), whereas the influence of dehydrocostus lactone was considerably higher by 7.3 ± 1.1 µg/mL (≙ 31.7 ± 4.8 µM). The commercial chemotherapeutic agents used as positive controls inhibited the growth of STS cell lines between 13.9 and 34.8 µM in the case of cisplatin and 0.7 and 2.5 µM in the case of doxorubicin. In control experiments, DMSO alone (0.5%) had no influence on the cells, regardless of incubation time.

To investigate the effects of costunolide and dehydrocostus lactone on cell cycle, SW-872, SW-982, and TE-671 cells were exposed to their respective IC_{50} of costunolide or dehydrocostus

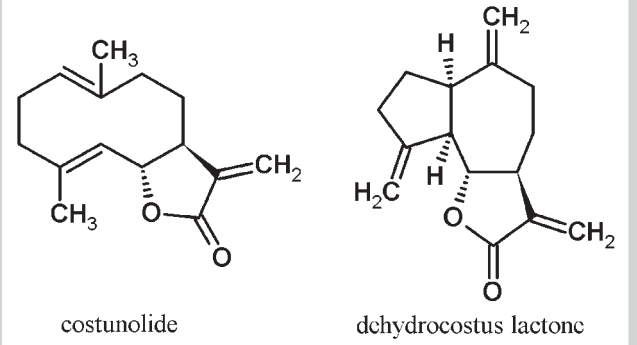
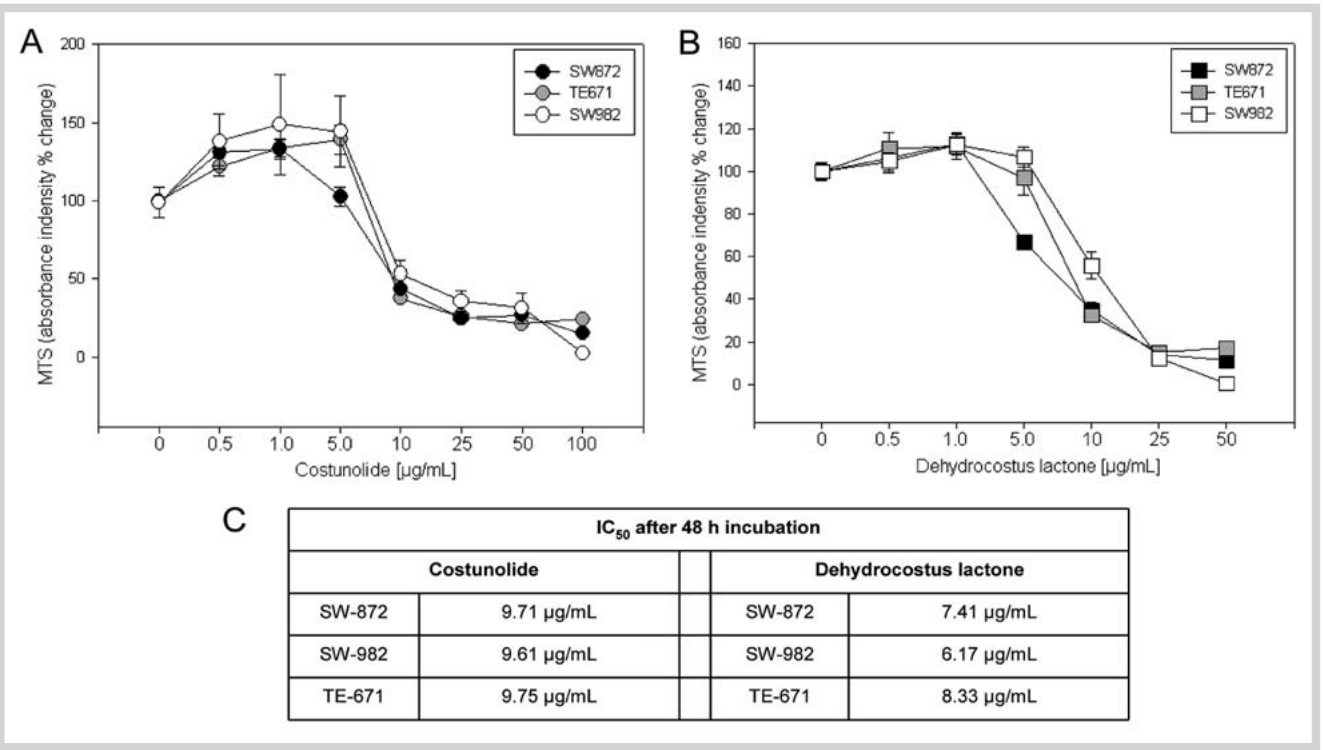


Fig. 1 Structures of active compounds identified in roots of *Saussurea lappa*.



C

IC_{50} after 48 h incubation			
Costunolide		Dehydrocostus lactone	
SW-872	9.71 µg/mL	SW-872	7.41 µg/mL
SW-982	9.61 µg/mL	SW-982	6.17 µg/mL
TE-671	9.75 µg/mL	TE-671	8.33 µg/mL

Fig. 2 Cell viability assay (MTS). Proliferation of SW-872, SW-982, and TE-671 cells when exposed to **A** 0.5–100 µg/mL costunolide and **B** 0.5–50 µg/mL dehydrocostus lactone for 48 h. Viability decreased dose-dependently. Data are represented as relative absorbance intensity of untreated cells (100%) (n = 12). IC_{50} values of both agents are listed in **C**.

lactone. Interestingly, when treated with costunolide, no significant changes in cell cycle distribution were detected compared to untreated control groups. In contrast, dehydrocostus lactone caused a decrease in the number of cells in the G1 phase after 48 and 72 h, which was accompanied by an increase of the number of S and G2/M phase cells. Measured and statistical data are summarised in **Tables 1** and **2**.

Apoptosis induction was investigated by annexin V/PI staining and quantified by measuring caspase 3/7 activity and caspase-3 cleavage. **Figs. 3**, **4** and **5** show the different effects of costunolide and dehydrocostus lactone regarding apoptosis. In adherent cells, the trypsinisation influences the annexin V staining in most cases and, therefore, the quantification due to the gating is difficult. Nonetheless, SW-872, SW-982, and TE-671 cells treated with dehydrocostus lactone for 48 h exhibited two populations with strong annexinV/PI staining. It can be concluded that these are late apoptotic populations. Costunolide seemed to have only a small effect on apoptosis induction (**Fig. 3**).

Active caspase-3, a marker for cells undergoing apoptosis, consists of a heterodimer of 17 and 12 kDa subunits, which is derived from a 32 kDa proenzyme [27]. Cleaved caspase-3 was measured by flow cytometry after 48 h exposure to the IC_{50} of costunolide and dehydrocostus lactone. The FACS histograms represent untreated cells (striated lines) versus costunolide-treated cells (checkered lines), and dehydrocostus lactone-treated cells (vertical stripped lines). In SW-872 cells, 16.93% cleaved caspase-3 was

Table 1 Cell cycle distribution of STS cell lines after 48 and 72 h exposure to costunolide (n = 3, mean ± SD).

Cell line	Treatment	Time	G_0G_1 (%)	S (%)	G_2/M (%)	Pi (%)	SPF (%)
TE-671	control	48 h	58.17 ± 0.91	25.21 ± 0.18	16.22 ± 6.40	41.83 ± 0.01	25.20 ± 0.01
	costunolide	48 h	53.52 ± 0.41	28.66 ± 0.16	18.22 ± 0.25	46.48 ± 0.01	28.26 ± 0.00
	control	72 h	86.28 ± 0.49	10.95 ± 1.97	2.77 ± 2.46	1.72 ± 0.00	10.95 ± 0.02
	costunolide	72 h	84.96 ± 0.86	12.39 ± 1.52	2.66 ± 2.38	15.04 ± 0.01	12.38 ± 0.02
SW-982	control	48 h	75.89 ± 0.14	13.13 ± 0.31	10.98 ± 0.17	24.11 ± 0.00	13.13 ± 0.00
	costunolide	48 h	66.93 ± 2.06	15.62 ± 0.74	17.46 ± 1.32	33.07 ± 0.20	15.62 ± 0.01
	control	72 h	76.63 ± 0.33	14.83 ± 0.45	8.54 ± 0.12	23.66 ± 0.00	14.83 ± 0.00
	costunolide	72 h	81.75 ± 0.19	8.72 ± 0.40	9.54 ± 0.20	18.25 ± 0.00	8.72 ± 0.00
SW-872	control	48 h	77.46 ± 0.58	14.14 ± 0.01	8.42 ± 0.59	22.54 ± 0.58	14.13 ± 0.01
	costunolide	48 h	75.16 ± 0.97	15.02 ± 0.32	9.83 ± 0.65	24.84 ± 0.97	15.02 ± 0.32
	control	72 h	88.60 ± 0.24	8.51 ± 0.27	8.89 ± 0.50	11.39 ± 0.00	7.53 ± 0.00
	costunolide	72 h	90.96 ± 0.05	6.15 ± 0.11	2.90 ± 0.05	9.04 ± 0.00	5.65 ± 0.00

* $P < 0.05$; $Pi = (S+G_2/M)/(G_0/G_1+S+G_2/M)$; $SPF = S/(G_0/G_1+S+G_2/M)$

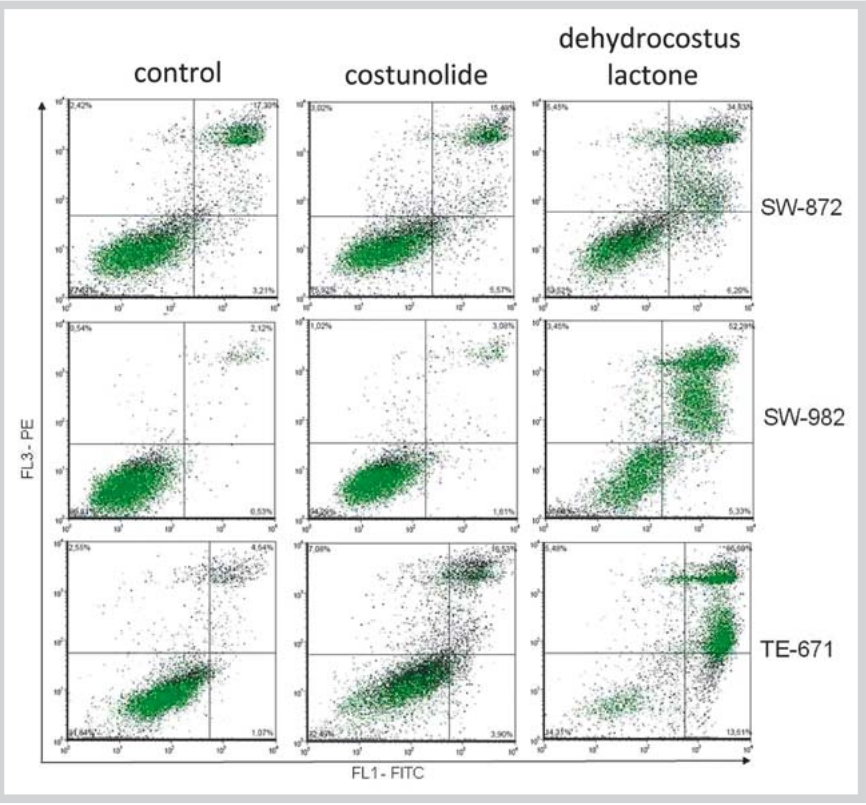


Fig. 3 Annexin V/PI apoptosis assay. SW-872, SW-982, and TE-671 cells treated with the IC_{50} concentration of dehydrocostus lactone for 48 h exhibited two populations with strong annexinV/PI staining. This indicates that these are late apoptotic populations. Treatment with the IC_{50} concentration of costunolide resulted in no apoptotic events.

detected in gated costunolide-treated and 96.82% in dehydrocostus lactone-treated cells (**Fig. 4A**). In contrast, the SW-982 cells showed almost no cleaved caspase-3 (10.72% and 12.93% of gated cells, respectively) (**Fig. 4B**). Also the dehydrocostus lactone-treated TE-671 cells showed a significantly higher proportion of cleaved caspase-3 (91.74%) in contrast to costunolide-treated cells (35.51%) (**Fig. 4C**). In addition, caspase 3/7 activity was measured by the Caspase-Glo® 3/7 assay in SW-872 (**Fig. 5A**), SW-982 (**Fig. 5B**), and TE-671 cells (**Fig. 5C**). It peaked after 24 and 48 h in SW-872 and TE-671 cells. Furthermore, the apoptotic induction by dehydrocostus lactone was confirmed by PARP cleavage in TE-671 cells (**Fig. 5D**).

Finally, we investigated the effects of costunolide and dehydrocostus lactone on mRNA expression levels of ABC transporters using real-time RT-PCR. Three major drug transporters (ABCB1/MDR1, ABCC1/MRP1, and ABCG2/BCRP1) were analysed. Costunolide did not significantly change the ABCB1/MDR1 expression. The expression levels of ABCC1/MRP1 and ABCG2/BCRP1 were significantly upregulated in costunolide-treated SW-872 and SW-982 cells (**Fig. 6A**). Dehydrocostus lactone treatment influenced the expression level of ABCB1/MDR1 and ABCG2/BCRP1 significantly in SW-872 and SW-982 cells, but not in the TE-671 cells. The ABCB1/MDR1 level was significantly downregulated in dehydrocostus lactone-treated SW-872 ($p = 0.0068$) and SW-982 cells ($p = 0.0024$). Dehydrocostus lactone also significantly lowered the expression levels of ABCG2/BCRP1 in the SW-872 ($p = 0.0058$) and SW-982 cells ($p = 0.0081$) (**Fig. 6B**). Furthermore, the ABCC1/MRP1 expression was downregulated in TE-671 cells ($p = 0.0014$), but not in the other cells lines. The downregulation of the ABCG2/BCRP1 and ABCB1/MDR1 transporter

Table 2 Cell cycle distribution of STS cell lines after 48 and 72 h exposure to dehydrocostus lactone (n = 3, mean ± SD).

Cell line	Treatment	Time	G_0G_1 (%)	S (%)	G_2/M (%)	Pi (%)	SPF (%)
TE-671	control	48 h	39.71 ± 0.48	42.24 ± 0.09	18.06 ± 0.40	60.30 ± 0.01	42.23 ± 0.00
	dehydrocostus lactone	48 h	27.69 ± 1.89	43.20 ± 0.42	29.12 ± 2.31	72.31 ± 0.02	43.20 ± 0.00
	control	72 h	76.99 ± 0.56*	14.14 ± 0.69*	8.87 ± 0.13*	23.00 ± 0.01	14.13 ± 0.01
	dehydrocostus lactone	72 h	56.26 ± 0.74*	28.36 ± 0.35*	15.38 ± 0.38*	43.74 ± 0.01	28.36 ± 0.00
SW-982	control	48 h	79.24 ± 1.65*	11.07 ± 0.48*	9.69 ± 1.16*	20.76 ± 0.02	11.07 ± 0.00
	dehydrocostus lactone	48 h	52.96 ± 0.44*	21.17 ± 1.11*	25.88 ± 1.55*	47.04 ± 0.00	21.16 ± 0.01
	control	72 h	80.80 ± 0.79*	11.31 ± 0.37*	7.90 ± 1.17*	19.20 ± 0.01	11.31 ± 0.00
	dehydrocostus lactone	72 h	55.71 ± 1.70*	19.13 ± 0.15*	25.17 ± 1.84*	44.29 ± 0.02	19.12 ± 0.00
SW-872	control	48 h	81.91 ± 0.32*	12.56 ± 0.18*	5.51 ± 0.50*	18.08 ± 0.00	12.58 ± 0.00
	dehydrocostus lactone	48 h	67.30 ± 0.00*	18.74 ± 0.80*	14.04 ± 0.80*	32.77 ± 0.00	18.74 ± 0.00
	control	72 h	91.09 ± 0.69*	6.84 ± 0.52*	2.08 ± 0.18*	8.91 ± 0.01	6.83 ± 0.01
	dehydrocostus lactone	72 h	50.81 ± 0.83*	33.25 ± 0.95*	15.95 ± 1.77*	49.20 ± 0.01	33.25 ± 0.01

* $P < 0.05$; $Pi = (S+G_2/M)/(G_0/G_1+S+G_2/M)$; $SPF = S/(G_0/G_1+S+G_2/M)$

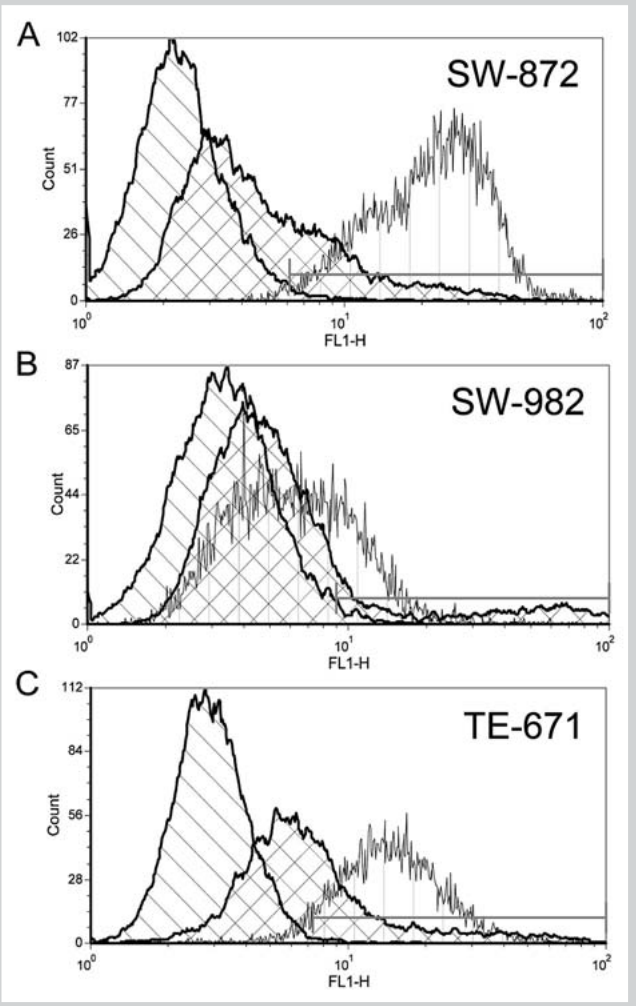


Fig. 4 Cleaved caspase-3 apoptosis assay. **A** SW-872, **B** SW-982, and **C** TE-671 cells were treated with the IC_{50} concentrations of costunolide or dehydrocostus lactone for 48 h. Cleavage of caspase-3 was detected by flow cytometry. The y-axis denotes cell counts, and the x-axis represents fluorescence intensity of FITC antibody. Striated lines represent untreated control cells, checkered lines represent costunolide treated cells, and vertical stripped lines represent dehydrocostus lactone-treated cells. Caspase-3 cleavage indicative for apoptotic cell death was especially found in SW-872 and TE-671 cells when exposed to dehydrocostus lactone.

expression in dehydrocostus lactone-treated SW-872 and SW-982 cells was confirmed using Western blot analysis (▶ **Fig. 7**).

Discussion

▼

Saussurea lappa Clarke is a well-known plant in Asia, where it is used in herbal medicine [17,18]. Previous *in vitro* and *in vivo* studies have shown that extracts of this plant have antiulcerative [28], anti-inflammatory [29], and antitumour properties [16,18, 30]. It has been shown that sesquiterpene lactones are the major components of the roots and exhibit various biological activities [20–22]. However, the effects of costunolide and dehydrocostus lactone in STS cells and their mechanisms of action are unknown and have yet to be analysed. Since chemotherapeutic agents in STS have been shown to be of limited efficacy, novel treatment strategies and therapeutic applications are needed and must be tested. In the present study, we have investigated the effects of costunolide and dehydrocostus lactone on cell viability, cell cycle, apoptosis, and ABC transporter expression levels in different human STS cell lines.

The inhibition of cell proliferation by such extracts and isolated compounds have already been shown in other cell types [18,31, 32] and suppressed telomerase activity and dehydrocostus lactone-induced apoptosis have been reported in some human cancer cell lines [33–38]. Our *in vitro* study has shown that costunolide and dehydrocostus lactone inhibited the growth of human STS cell lines dose- and time-dependently. IC_{50} values ranged from 41.3 µM to 42.0 µM in the case of costunolide, and from 26.8 to 36.1 µM in the case of dehydrocostus lactone. Furthermore, we analysed the influence on cell cycle distribution. Treatment with costunolide did not alter the cell cycle distribution after 48 and 72 h. In contrast, dehydrocostus lactone caused a significant decrease of cells in the G1 phase and an increase at S and G2/M phases. It has also been shown that *Saussurea lappa* extracts induce G2 growth arrest and apoptosis in gastric cancer cells [31,32]. Furthermore, an effect of dehydrocostus lactone on cell cycle distribution was reported in MDA-MB-231 breast and SK-OV-3 ovarian cancer cells [42,43]. Both types of cancer cells were also arrested at the G2/M interface. Cells can be temporarily arrested to allow for cellular damage to be repaired, or the arrest may result in the activation of pathways leading to programmed cell death [44,45]. We detected not only the G2/M arrest but also morphological changes indicative for apoptosis such as detached cells, membrane blebbing, and rounded morphology. Additional annexin V staining also showed a clear hint of apoptosis. Annexin V-FITC is used to quantitatively determine the percentage of cells within a population that are undergoing apoptosis. It relies on the property of cells to lose membrane asymmetry in the early phases of apoptosis. Propidium iodide is a standard flow cytometric viability probe and used to distinguish viable from nonviable cells. We found significant late apoptotic populations in dehydrocostus lactone-treated STS cells, with TE-671 showing the strongest effect. To substantiate the apoptotic effect, caspase 3/7 activity was measured and could be confirmed by FACS caspase-3 measurements. Activation of caspases by dehydrocostus lactone, especially those involved in the intrinsic apoptotic pathway, have also been reported for other types of cancer cells [36,39–41] but not yet for STS cells.

Some plant extracts of Chinese herbs were found to inhibit MDR efflux pumps [46], which mediate multidrug resistance in many types of cancer. Therefore, we analysed the mRNA expression of major drug transporters (ABCB1/MDR1, ABCC1/MRP1, and ABCG2/BCRP1) of the ABC transporter family, which may provide highly suitable targets for clinical cancer therapy. ABCB1/MDR1 and ABCG2/BCRP1 were significantly downregulated in SW-872 and SW-982 cell lines after treatment with dehydrocostus lactone indicating that this compound has a certain potential to circumvent multidrug resistance in these cells.

In summary, we have shown that costunolide and dehydrocostus lactone inhibited the growth of STS cells *in vitro*. Our data indicate that particularly dehydrocostus lactone is a potential therapeutic lead for the development of novel STS treatment strategies. So far, it seems to induce caspase-dependent apoptosis and may circumvent cancer drug resistance by reducing the expression of ABC transporters. Our findings provide the basis for further investigations to clarify novel treatment regimens for drug-resistant tumours.

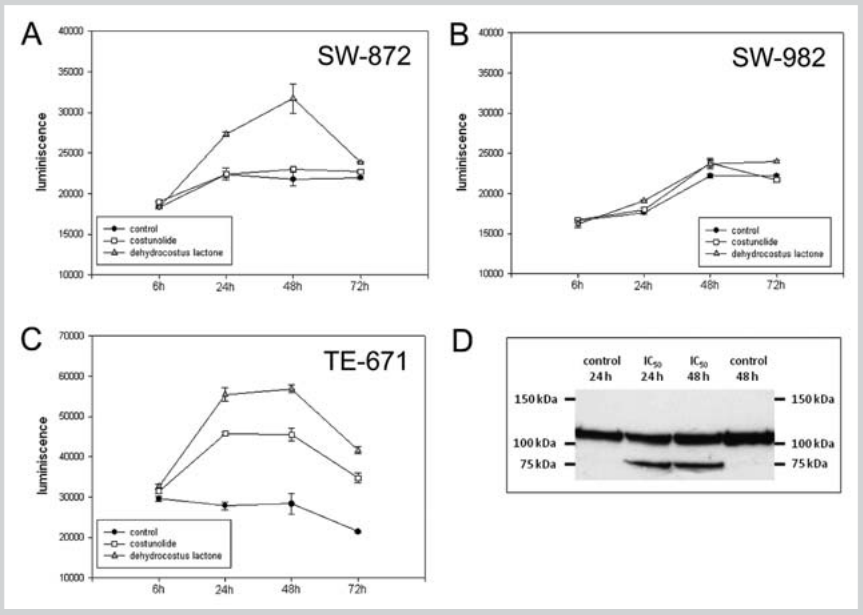


Fig. 5 Caspase-Glo® 3/7 apoptosis assay. The activity of caspase-3/7 was measured in **A** SW-872, **B** SW-982, and **C** TE-671 cells by the Caspase-Glo® 3/7 assay. Dehydrocostus lactone-treated SW-872 and TE-671 cells showed a significantly higher level of cleaved caspase-3 and active caspase-3/7 compared to costunolide-treated cells. **D** Whole cell lysates of cells exposed to dehydrocostus lactone were subjected to Western blot analysis using anti-PARP antibody. The results confirmed the supposed apoptosis induction.

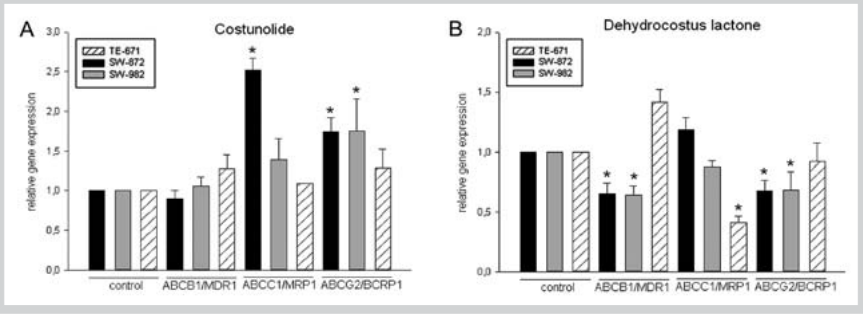


Fig. 6 Relative mRNA expression of ABC transporter genes measured by real-time RT-PCR. Expression levels of ABCB1/MDR1, ABCC1/MRP1, and ABCG2/BCRP1 are shown in untreated (control), **A** costunolide-, and **B** dehydrocostus lactone-treated STS cell lines (n = 8). The expression levels were normalised (ΔCt) to the expression of *β*-actin, GAPDH, and hprt-n as an internal control and compared to the corresponding ΔCt (ΔΔCt) of control cells. * $P < 0.05$.

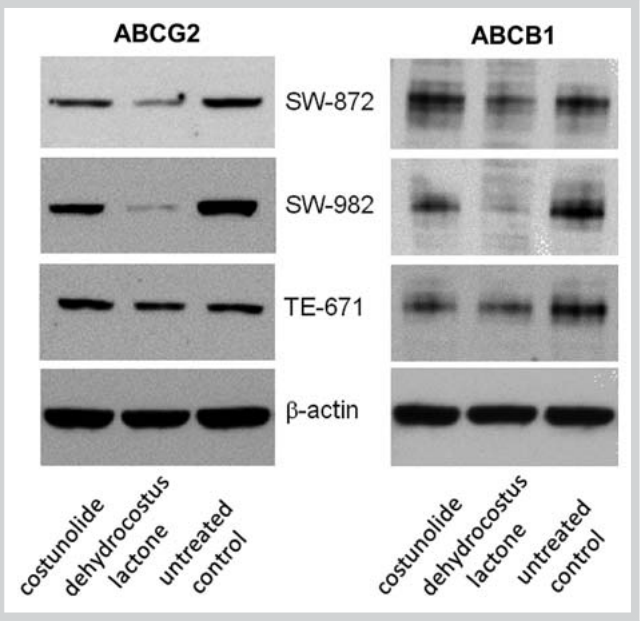


Fig. 7 Western blot analysis of ABC transporter genes. The total protein analysis confirmed the downregulation of the ABCG2/BCRP1 and ABCB1/MDR1 transporters in dehydrocostus lactone-treated SW-872 and SW-982 cells.

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Conflict of Interest

There are no conflicts of interest.

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