

Boswellic acid acetate induces differentiation and apoptosis in highly metastatic melanoma and fibrosarcoma cells

Wanzhou Zhao, PhD^{a,*}, Frank Entschladen, PhD^b, Hongyan Liu, BS^a,
Bernd Niggemann, MD^b, Qicheng Fang, PhD^a, Kurt S. Zaenker, MD^b, Rui Han, PhD^a

^a Institute of Materia Medica, Chinese Academy of Medical Sciences & Peking Union Medical College (CAMS & PUMC),
No.1 Xiannong Tan Street, Beijing 100050, China

^b Institute of Immunology, University Witten/Herdecke, Stockumer Street 10, 58448 Witten, Germany

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Abstract

The aim of the study was to investigate the antitumor and/or preventive effect of BC-4, an isomeric compound isolated from the plant *Boswellia carteri* Birdw. containing alpha- and beta-boswellic acid acetate in 1:1, MW 498.3. We used the MTT (3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide) assay to study the growth inhibition activity of BC-4. Tumor cells migration within a three-dimensional collagen matrix was recorded by time-lapse videomicroscopy and computer-assisted cell tracking. Topoisomerase II was isolated from mouse melanoma B16F10 cells and its activity was determined by its ability to cut plasmid pBR322 DNA. The secretion and activity of matrix metalloproteinases (MMPs) from human fibrosarcoma HT-1080 cells were determined by gelatin zymography. BC-4 was a cytostatic compound and could induce the differentiation of B16F10 mouse melanoma cells, blocked the cell population in G1 phase and inhibited topoisomerase II activity. The G1 phase population of B16F10 cells was increased from 57.4 to 87.7%, while S phase population was reduced from 33.3 to 5.9% after treatment with BC-4 at 25 μ M concentration for 48 h. BC-4 also inhibited the migration activity of B16F10. BC-4 could induce apoptosis of HT-1080 cells, as proved by acridine orange fluorescence staining, Wright–Giemsa staining, electromicroscopy, DNA fragmentation and flow cytometry. BC-4 inhibited the secretion of MMPs from HT-1080 cells, too. In conclusion, if it turns out that BC-4 is a well tolerated substance, exhibiting no significant toxicity or side effects, being evaluated currently in China, BC-4 is a good candidate for the prevention of primary tumor, invasion and metastasis.

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Keywords: Boswellic acid acetate; Differentiation; Apoptosis; Matrix metalloproteinases; Migration; Chemoprevention

1. Introduction

A difficult and complex area of plant research is devoted to a plant with the general title “frankincense”. It is accepted that frankincense is a member of the family Burseraceae, and the genus *Boswellia*. From all of the sources accessed, a few front runners emerged: *Boswellia sacra*, *Boswellia serrata* and *Boswellia carteri*. Frankincense was commonly used for medicinal purposes. Pliny the Elder (1st century), used frankincense as an antidote to hemlock poisoning. The Iranian physician Avicenna (10th century) thought that it was good for body ailments such as tumors, vomiting, dysentery and fevers. *Boswellia carteri* Birdw. is used in traditional Chinese medicine (TCM) which is a remedy for activat-

ing blood circulation, relieving pain against leprosy, cancer, gonorrhea and carbuncles, and as an astringent. Boswellic acid acetate (BC-4) is one of several active principles isolated from the resin of this herb. There are two isoforms of boswellic acid acetate, α -boswellic acid acetate (BC-4-I) and β -boswellic acid acetate (BC-4-II) and both do have a pentacyclic triterpene structure.

Boswellic acid derivatives show a strong antiinflammation effect *in vivo* and this effect has been proved to be due to the inhibition of 5-lipoxygenase (5-LO) [1]. It has also been reported that 5-LO inhibitors inhibit the growth of non-small cell lung cancer [2]. Furthermore, lipoxygenase inhibitors demonstrate an activity as cancer chemopreventive agents [3]. Based on the screening in HL-60 cells for differentiation inducers, BC-4 was identified as a potent differentiation inducer [4]. We have reported that BC-4 can induce myelocytic leukemia cell differentiation at low concentration and at higher concentrations apoptosis in leukemia cells [5]. In addition to hematologic malignancies, an apoptotic effect

* Corresponding author. Present address: Department of Immunology, Roswell Park Cancer Institute, Elm & Carlton Streets, Buffalo, New York 14263, USA. Tel.: +716-845-1750; fax: +716-845-8906.
E-mail address: wanzhou.zhao@Roswellpark.org (W. Zhao).

on malignant glioma cells by boswellic acids was observed [6]. Here, we reported that BC-4 induces differentiation in mouse melanoma cells and triggers apoptosis in human fibrosarcoma cells.

2. Materials and methods

2.1. Cell lines and culture conditions

Human oropharyngeal epidermoid carcinoma KB, human colon carcinoma cell line HCT-8, human ovarian-adenocarcinoma cell line A2780, mouse melanoma cells B16F10, human fibrosarcoma cell line HT-1080, are permanently maintained as cell lines in the Department of Pharmacology I, Institute of Materia Medica, CAMS & PUMC. Cells are kept in RPMI 1640 medium containing 10% fetal bovine serum, streptomycin (100 µg/ml), and penicillin (100 units/ml) and were cultured at 37 °C in a humidified atmosphere of 5% (v/v) CO₂.

Boswellic acid acetate is kindly provided by Prof. Jinyun Zhou (Institute of Materia Medica, CAMS & PUMC). It is a pure colorless crystalline compound. HPLC analysis indicates that the α- and β-boswellic acid acetate are present in BC-4 in 1:1 ratio with an average molecular weight 498.3. BC-4 was dissolved in ethanol as a stock solution of 10 mg/ml.

2.2. Proliferation assay (MTT)

The KB, HCT-8, A2780 cells (1×10^4 per ml) were used for MTT assay. MTT (3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide) was purchased from Sigma. Into 96-well microculture plates 100 µl of cell suspension was seeded and allowed to adhere for 24 h before BC-4 was added. Each tumor cell line was exposed to BC-4 at log concentrations from 10 mg/ml to 1 µg/ml for 72 h. The experiments were performed in triplicates. At the end of the exposure time, the medium was removed and 100 µl of MTT solution (0.4 mg MTT/ml in serum free medium) was added to each well. The plates were incubated at 37 °C for 4 h. Then the solution was removed and 150 µl DMSO was added to each well. After shaking for 4 min, the optical value was read by means of a BIO-RAD (Hercules, CA) Model 550 microplate reader at a wavelength of 550 nm. The growth inhibition of BC-4 on these tumor cells was expressed as IC₅₀ (concentration of the drug reducing the proliferation rate by 50% compared to control values).

2.3. Cell viability assay

Cells were initially seeded at 1.75×10^5 cells per flask. The concentration of 12.5 and 25 µM BC-4 were added 16 h after initial cell seeding. The cells were allowed to grow for 5 days. Total cell count was done by a hemocytometer. Cell viability was determined by staining the cells with 0.25%

trypan blue, and the fraction of unstained cells was considered as the surviving fraction. Cellular morphology was documented by microscopic photographs. The state of differentiation was assessed (i) by the change in morphology, (ii) the growth potentials within the context of sustained cell viability. It is said, an agent is cytotoxic, if there is a substantial growth inhibition accompanied by a drastic decrease in cell viability; in contrast, a substance is cytostatic, if the proliferation is decreased, but the cell viability within the population is still maintained at a high level.

2.4. Assay of melanin content

The content of melanin was assayed according to the procedure described by Meyskens [7]. Cells were plated in flasks containing 10 ml of RPMI 1640 medium. Twenty-four hours after plating, cells were removed from the dish by 0.25% trypsin, and the number was counted. After 1000 rpm centrifugation for 10 min, the cells were washed twice with phosphate-buffered saline (pH 7.0) and 10^6 cells were dissolved in 1.0 ml of 1N NaOH and 10% dimethyl sulfoxide for 30 min. Thereafter the absorbance at 470 nm was recorded.

2.5. 3-D collagen preparation and tumor cell migration assay

3-D collagen matrices were prepared as described [8]. Briefly, 50,000 cells suspended in PBS containing BC-4 were mixed with a collagen solution consisting of 1.67 mg/ml type I bovine dermal collagen (Vitrogen 100, Cella, Strassen, Luxemburg) in Earle's modified Eagle's minimal essential medium (MEM) adjusted to pH 7.4. Then, 150 µl of this cell suspension was filled into a small chamber and allowed to polymerize for 20–30 min (37 °C, 5% CO₂).

Cell migration was analyzed according to [8]. Briefly, cells incorporated within the collagen lattices were visualized on a conventional inverted microscope. Time-lapse videomicroscope was employed to record the movement. Subsequently, the paths of individual cells were reconstructed from the recorded films by computer-assisted cell tracking. The time-lapse video movie was displayed on a computer screen. From the first frame of the time-lapse sequence 30 cells were randomly selected, giving a non-biased, representative samples of the cell population. Subsequently, the movements of each of these cells were individually followed with a trackball. Every 2 min (real time of the time-lapse film) the *x*–*y* coordinates of the screen-pointer were registered by the computer. Plotting the *x*–*y* data on the screen reconstructed the paths of individual migrating cells. The vector length between two *x*–*y* coordinates was used as a direct measure of distance. Analyzing the corresponding vectors in each of the 30 paths simultaneously gave access to the percentage of migrating cells in a population (i.e. vector > 0) at a given time point (activity).

2.6. Analysis of cell cycle progression

Preparation of cells for analysis was done as described previously [9]. Briefly, cells were labeled with 30 μ M BrdUrd for 15 min and then harvested by trypsinization, counted, and washed with PBS. Cells were then fixed in cold 70% ethanol at a concentration of 1,000,000 cells/ml. Fixed cells were stored at 4 °C for up to 10 days. The BrdUrd/DNA signal was analyzed with the use of a Becton Dickinson FACScan (Bedford, MA) flow cytometer with 15 mW excitation at 488 nm. The FITC-conjugated anti-BrdUrd antibody was purchased from Becton Dickinson. The FITC signal was collected through a 530/30 band pass filter on FL1, and the propidium iodide (PI) signal was assessed through a 650 long pass filter on FL3. The software used for acquisition and analysis was Lysis II (Ver. 1.1; Becton Dickinson). Doublets were excluded by analysis of two-dimensional plots of fluorescence pulse width versus area of the PI signal. The percentage of the events was calculated by the Lysis II program.

2.7. Measurement of topoisomerase II catalytic activity

2.7.1. Extraction of topoisomerase II

All procedures were carried out at 4 °C. Fresh PMSF was added to all buffers at a final concentration of 1 mM. B16F10 cells were treated with 12.5 and 25 μ M concentration of BC-4 for 24 h. Then cells were trypsinized and centrifuged at 5000 rpm for 10 min. The pellet was resuspended in 30 ml of PBS and sedimented at 4000 rpm for 10 min. The pellet was washed twice with PBS and centrifuged again. After the final wash, the cells were resuspended in 10 ml of 10 mM Tris (pH 7.5), 1.5 mM $MgCl_2$, 10 mM NaCl and allowed to sit at 4 °C for 10 min. A nonionic detergent (1 ml of 10% Nonidet P-40) was added and the mixture gently triturated and finally left at 4 °C for 15 min. The cells were then homogenized and centrifuged at 2500 rpm for 10 min, and the pellet was resuspended in 2 ml of buffer A (50 mM Tris (pH 7.5), 25 mM KCl, 2 mM $CaCl_2$, 3 mM $MgCl_2$, and 0.25 M sucrose). The nuclei thus obtained were layered over 0.6 ml of buffer B (buffer A with 0.6 M sucrose) and sedimented at 7000 rpm for 10 min. The pellet was resuspended in 2 ml of buffer C (buffer A without $CaCl_2$ but with 5 mM $MgCl_2$), and centrifuged at 7,000 rpm for 10 min, and finally resuspended in 0.3 ml of buffer D (same as buffer C but without sucrose). To this solution 30 μ l of 0.2 M EDTA (pH 8.0), and 0.66 ml of buffer E (1 mM Tris (pH 7.5), 2 mM EDTA, 1 mM DTT, 0.53 M NaCl, and 20% glycerol (v/v)) were added. This mixture was gently triturated, left at 4 °C for 30 min, and centrifuged at 19,000 rpm for 20 min. The supernatant from the last centrifugation was the topoisomerase II preparation. The protein concentration was determined by the Bradford method. The enzyme solution was diluted with an equal volume of glycerol. BSA and PMSF were added to final concentrations of 1 mg/ml and 0.5 mM, respectively. The enzyme was stable at –20 °C for 4–5 days.

2.8. Cleavage of plasmid pBR322 DNA

Assays for topoisomerase II mediate DNA cleavage followed the method as described previously [10], a slight modification was done. The reaction tube contained 1 μ g of pBR 322 DNA, and topoisomerase II (total protein preparation 145 μ g). After incubation at 37 °C for 10 min, the reaction was stopped by the addition of 5 μ l of 10% SDS and 1 mg/ml proteinase K. The mixture was allowed to stand at 37 °C for 30 min. The samples were then loaded onto 0.7% agarose gels and subjected to electrophoresis. Gels were stained with 0.5 μ g/ml ethidium bromide and observed under UV light.

2.9. Gelatin zymography

2.9.1. Secretion assay

HT-1080 cells (2.5×10^5) were cultured overnight in 24-well plates. The cells were rinsed twice with Ca^{2+} and Mg^{2+} -free PBS and incubated in serum-free RPMI 1640 medium. One hour later, the cells were rinsed twice again and incubated in serum-free RPMI 1640 medium or medium containing BC-4. After a 24 h incubation, the supernatant was collected, and centrifugation at 3000 rpm for 10 min at 4 °C. The cell-free medium was used for detection of gelatinase activity. Gelatin zymographs were performed according to the method as reported [11] with some modifications. Briefly, samples were applied to SDS-polyacrylamide gels (10%, w/v) containing 0.1% (w/v) gelatin. After electrophoresis the gels were rinsed twice (30 min and 1 h each) in 2.5% Triton X-100 to remove SDS, and incubated for 18 h at 37 °C in incubation buffer (50 mM Tris-HCl, pH 7.5, containing 10 mM $CaCl_2$, 1 μ M $ZnCl_2$, 200 mM NaCl).

2.9.2. Activity assay

In the assay of BC-4 on gelatinolytic activity, BC-4 was added in the incubation buffer. The gelatin gels were then stained with 0.1% coomassie blue R-250, containing 10% acetic acid, 10% isopropanol, destained in 10% acetic acid, 10% isopropanol, and dried. Enzyme-digested regions were identified as white bands against a blue background.

2.10. Apoptosis analysis

2.10.1. Chromatin condensation assay

For observation of chromatin condensation, BC-4 treated HT-1080 cells were kept in a PBS suspension. For this 5 μ l of 0.01% acridine orange/PBS (pH 6.8) solution was added into 95 μ l cell suspension. Then the mixture of 10 μ l cell suspension was added on a slide and covered with a slip. The condensed chromatin parts appeared under an Olympus Fluorescence Microscope as “apoptotic bodies”. Cells from the same preparation were fixed and stained with Wright and Giemsa for the observation of condensed and fragmented nuclei.

2.10.2. Electron microscopical study

Cells were trypsinized and centrifugated at 600 rpm for 5 min. Cell pellets were fixed in 1 ml of ice cold 95% ethanol for 12 h, in 2.5% glutaraldehyde in PBS for 1 h, then in 1% osmium tetroxide in PBS for 1–2 h, dehydrated by ethanol, and embedded in Epon. Ultrathin sections were contrasted with uranyl acetate and hydroxide and examined with a Japan Electron Optics Laboratory JEM 200CX electron microscope.

2.10.3. Flow cytometry and DNA fragmentation

HT-1080 cells were treated with BC-4 and the apoptotic cells were identified either by flow cytometry [12] or by the presence of DNA fragmentation as described previously [13]. In the latter case, treated and untreated cells were washed twice with PBS containing 0.2 mM EDTA (pH 6.8) at ambient temperature and lysed in 10 mM EDTA, 50 mM Tris-HCl (pH 8.0), 0.5% SDS, and 0.5 mg/ml proteinase K. Cell lysates were then treated with 0.15 mg/ml RNase for 1 h at 37 °C and then with 1.0 mg/ml proteinase K at 37 °C for at least 1 h. Loading buffer (10 mM EDTA, 1% (w/v) low melting point agarose, 0.25% (w/v) bromophenol blue, and 40% (w/v) sucrose) was then added (10% final concentration). Heated (70 °C) samples were loaded onto presolidified 1.7% (w/v) agarose gels containing 0.5 µg/ml ethidium bromide. Agarose gels were run at 65 V for 10 min and then at 25 V overnight. Gels were photographed under UV light.

3. Results

3.1. Growth inhibition (cytostasis) and differentiation effects of BC-4 on KB, HCT-8, A2780 and B16F10 cells

In Fig. 1, the chemical structure of BC-4 is given. There exist two isoforms, BC-4-I (α) and BC-4-II (β). BC-4 showed a marginal growth inhibition potential on KB, HCT-8, A2780 cells of IC_{50} 10.71–13.39 µM (data not shown).

BC-4 also inhibited the growth of B16F10 mouse melanoma cells in culture in a dose and time-dependent fashion (Fig. 2, top). The graph shows the cells in culture for 5 days; the growth kinetics are recorded for living cells, only those cells, which exclude the staining dye. During the culture period in an exponential growing phase, B16F10 (without BC-4) exhibited round cells and many clumps were observed at confluency (Fig. 2A). The treatment of melanoma cultures with BC-4 caused dramatic changes in morphology which were also dose-dependent. The effect became visible as early as 24 h after beginning of BC-4 treatment. At a concentration of 12.5 µM, cells started to get elongated (Fig. 2B). At a higher concentration (25 µM) cells were large, arranged in parallel at several locations and markedly elongated than before (Fig. 2C). Control cultures treated with similar amounts of pure solvent for BC-4 did not cause any significant change in morphology.

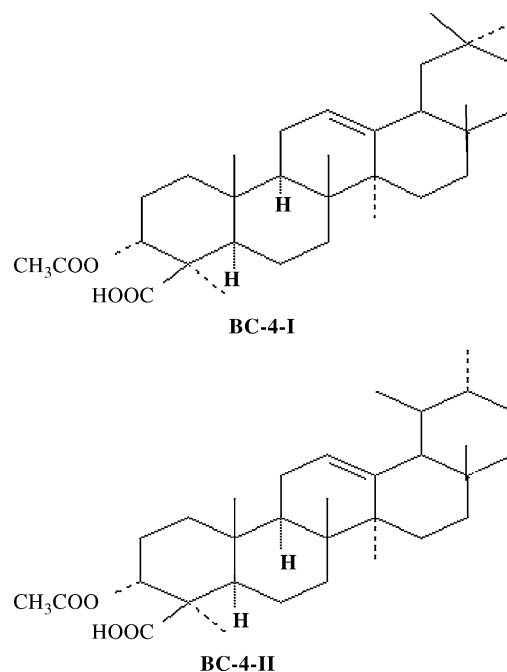


Fig. 1. Chemical structure of BC-4-I (α -boswellic acid acetate, 3a-hydroxyl-olean-12-en-24-oic acid acetate or 3a-acetoxy-plean-12-en-24-oic acid) and BC-4-II (β -boswellic acid acetate, 3a-hydroxyl-ur-12-en-24-oic acid acetate or 3a-acetoxy-urs-12-en-24-oic acid) as they are present in BC-4.

The melanin content of B16F10 cells in untreated cultures exhibited a low baseline. The melanin content increased about 16-, 20-fold, respectively, when the melanoma cells were treated with BC-4 at 50 µM for 24 or 72 h. (Fig. 3A). Besides the obvious change in morphology and melanin content, both of which were differentiation marker for B16F10 cells, the cell migration of B16F10 was significantly inhibited (Fig. 3B).

After treatment of B16F10 cells with BC-4 at 25 µM up to 48 h, the cell population in G1 phase increased steadily from 57.4 (control, Fig. 4A) to 87.7% (Fig. 4D), whereas the cell population in S phase decreased concomitantly from 33.3 (control, Fig. 4A) to 5.9% (Fig. 4D). At the same time, the topoisomerase II activity from B16F10 melanoma cells was also inhibited after treatment with BC-4 at 25 µM for 24 h (data not shown).

3.2. Inhibition of MMPs secretion by human fibrosarcoma HT-1080 cells

Fig. 5 demonstrates the effect of BC-4 treated human fibrosarcoma cells on the secretion of metalloproteinase 2 and 9. Lane one is the control, lane 2 the solvent for BC-4. The pictures revealed an intensive lysate activity for gelatin. In lanes 3–6 the cells were treated by increasing BC-4 concentrations which are accompanied by an almost diminished secretion activity of MMP-9 and MMP-2.

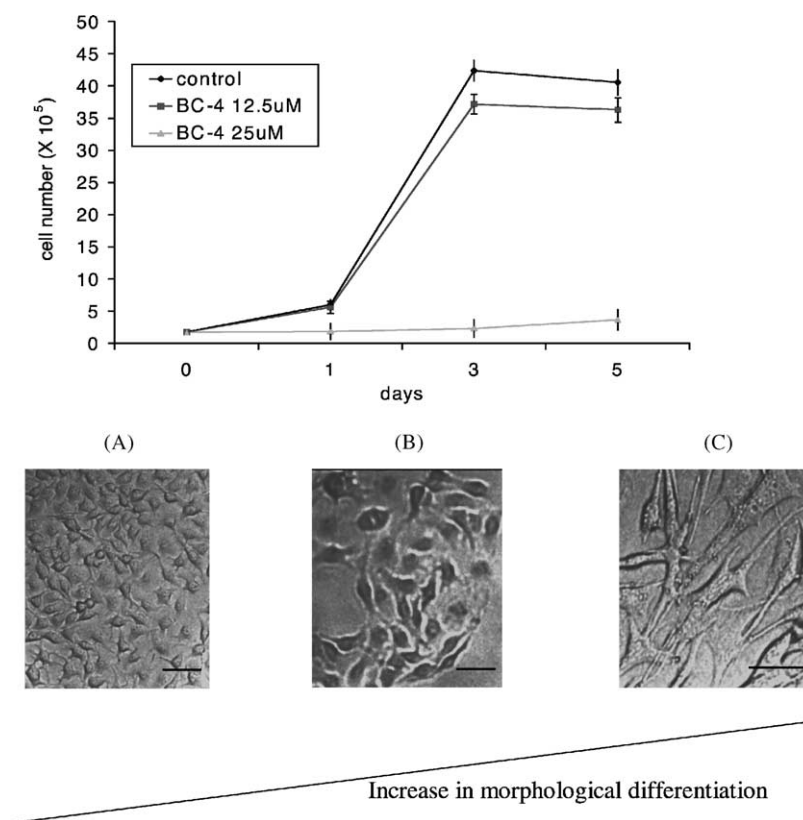


Fig. 2. Effect of BC-4 on survival of mouse melanoma cells B16F10. Cell survival was determined by staining cells with 0.25% trypan blue. Only those cells were counted which excluded the dye. Photomicrographs were taken after 24 h treatment of BC-4. (A) Control culture contains B16F10 cells rounded up or in clumps; (B) B16F10 treated with 12.5 μ M; (C) B16F10 treated with 25 μ M BC-4. Cultures treated with BC-4 exhibited a dramatic change in morphology. Bars 50 μ m.

3.3. Five different settings to evaluate apoptosis induction by BC-4 in human fibrosarcoma HT-1080 cells

BC-4 can induce apoptosis in HT-1080 cells, as proved by acridine orange fluorescence staining (Fig. 6A), Wright–Giemsa staining (Fig. 6B), electron microscopy observation (Fig. 6C). The morphological pictures nicely show the appearance of apoptotic bodies by light and electron microscopic techniques. The DNA laddering is given in Fig. 6D. The DNA laddering appeared as dispersed laddering. The flow cytometry (Fig. 6E) also suggested the induction of apoptosis by revealing a sub-G1 peak of PI stained cells. The apoptosis effect was dose and time-dependent. Apoptotic cells increased from 0.42 (control) to 59.3% after treatment with BC-4 at 50 μ M for 36 h, while treated with BC-4 at 75 μ M for 24 h the apoptotic cells increased to 76.5%.

4. Discussion

In this study, we have tested a panel of human cancer cell lines for their sensitivity to BC-4 in respect to growth inhibition, differentiation and apoptosis induction and ask-

ing the question whether BC-4 exhibits cytotoxic or cytostatic effects. The results strongly suggest that BC-4 is a cytostatic compound. The results also prove that BC-4 has a strong effect on inducing differentiation in mouse melanoma cells B16F10 and apoptosis in human fibrosarcoma cells HT-1080.

After treatment with BC-4, the proliferation rate of B16F10 cells was inhibited, also the cells were still alive, because only those cells were counted which excluded the dye. Besides this effect, the morphology of melanoma cells changed obviously. Since melanocytes deliver melanin to keratinocytes via long dendritic processes, the formation of dendritic-like structure indicates cellular differentiation in melanoma cells. It has been suggested that melanoma cells are considered to be differentiated when their dendrites are at least two times longer than the diameter of cytoplasm [14]. In our study, mouse melanoma B16F10 cells treated with BC-4, pronounced dendritic processes appeared after 1 day of treatment and persisted through the remaining course of induction; BC-4 has to be present in the medium throughout the observation period. This long dendritic processing appeared in 25 μ M BC-4 treated cells, and the length of these dendrites were approximately 10-time longer than the diameter of control cytoplasm.

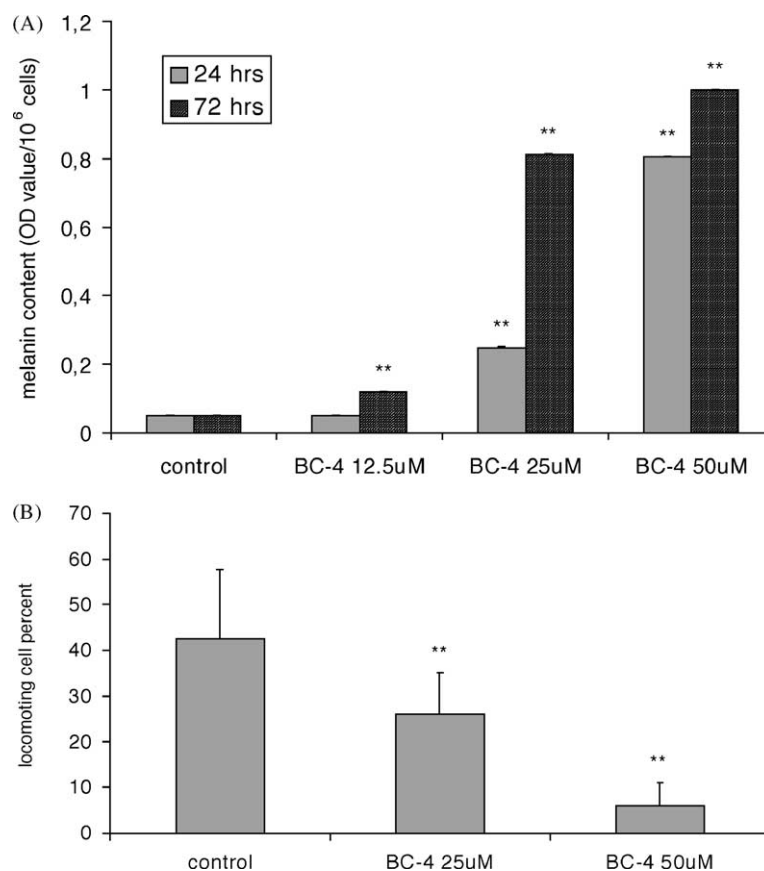


Fig. 3. (A) Effect of BC-4 on the melanin content of mouse melanoma cells B16F10. The melanin content increased about 16-, 20-fold, respectively, when the melanoma cells were treated with BC-4 50 μM for 24 and 72 h, respectively. Data represents the mean of three independent biological determinations. (B) Migration ability of B16F10 mouse melanoma cells. The cell locomotion in the collagen matrix containing BC-4 was recorded by time-lapse videomicroscopy. Thirty cells were randomly selected and computer-assisted cell tracking was used to analysis locomoting cell percent. Bars, \pm S.D.; ** $P < 0.01$ vs. control cells. The statistical significance of differences in terms of melanin content and locomotion cell percent between control and BC-4 treated groups was evaluated by Student's *t*-test.

B16F10 is an aggressive and highly metastatic cell line. One feature of metastasis is the induction of cell locomotion, without cell locomotion no metastasis formation is possible. The B16F10 melanoma cells do show a high grade of spontaneously locomotion when they are seeded into 3-D collagen matrix. After treatment with BC-4 the B16F10 melanoma cells decrease their mobility and increase their melanin content as well as their cell processes. These are elegant surrogate markers *in vitro* of re-differentiation by gaining a benign phenotype. However, this switch from a malignant to a more benign appearance has to be validated *in vivo* by showing that these cells, recruiting the re-differentiation markers under the command of BC-4 are less metastatic than the wild type cells.

DNA topoisomerase II is present in both eukaryotes and prokaryotes and exists as a dimer with a subunit molecular mass of 131–180 kDa. This enzyme catalyzes DNA topoform interconversions by introducing a transient enzyme-bridged, double-strand break in one of the two crossing DNA segments. Several investigators have suggested that topoisomerase II is intimately involved in a

variety of intracellular genetic processes, which involve DNA topological isomerization, and drug-induced tumor cell differentiation as explored by our experiments. Recent data showed that acetyl-11-keto-beta-boswellic acid inhibited topoisomerase I from calf thymus at concentrations of $\geq 10 \mu\text{M}$ [15]. Acetyl-boswellic acids were reported as novel catalytic inhibitors of human topoisomerases I and II α , with K_d values of 70.6 and 7.6 nM, respectively [16]. This is an interesting pharmacodynamic observation. Our results point into a similar direction of topoisomerase II inhibition, however, with the exception that we observed a more indirect effect *in vitro*. We incubated the topoisomerase II preparation with BC-4 *in vitro* and did not get an inhibition of enzyme activity up to the concentration of 50 μM BC-4 (data not shown). Although this result might be in contradiction to the reported [16], we can not exclude the possibility of error that in our enzyme preparation are inhibitors for the BC-4 activity which are not active in living cells.

HT-1080 is also a highly invasive cell line, which secretes high level of metalloproteinases. Our experiments

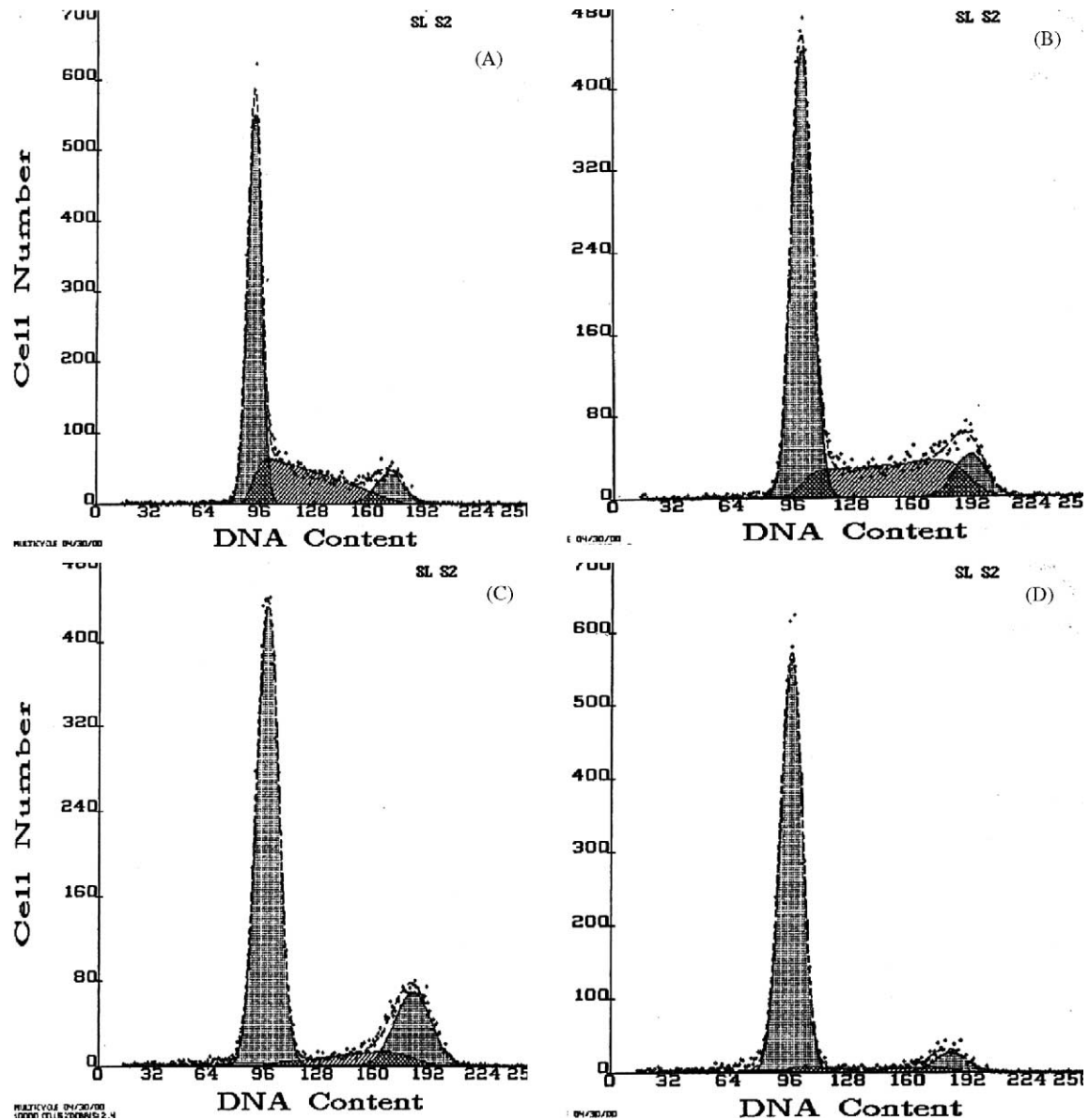


Fig. 4. Cell cycle analysis of B16F10 mouse melanoma cells after treatment with 25 μ M BC-4 for different time. The population with the BrdUrd-negative signal and 2N DNA content detected by PI is in G1. The population with the BrdUrd-positive signal and 4N DNA content is in G2-M. Those cells with BrdUrd-positive signal are in S phase. (A) Control, G1 = 57.4%, S = 33.3%; (B) BC-4 for 24 h, G1 = 62.0%, S = 28.9%; (C) BC-4 for 36 h, G1 = 72.3%, S = 8.5%; (D) BC-4 for 48 h, G1 = 87.7%, S = 5.9%. Results shown are from one of three representative experiments.

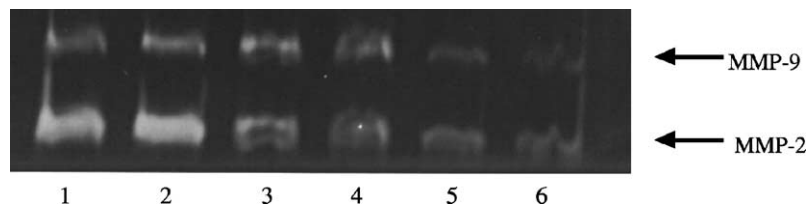


Fig. 5. Gelatin zymography was used to determine the effect of BC-4 on the secretion of MMPs from human fibrosarcoma HT-1080 cells. Lane 1, control; lane 2, solvent; lanes 3–6, cells were treated with BC-4 3.125, 6.25, 12.5, 25 μ M, respectively. The concentrations of BC-4 for MMPs secretion assay were tested to have no effect on the viability of HT-1080 cells.

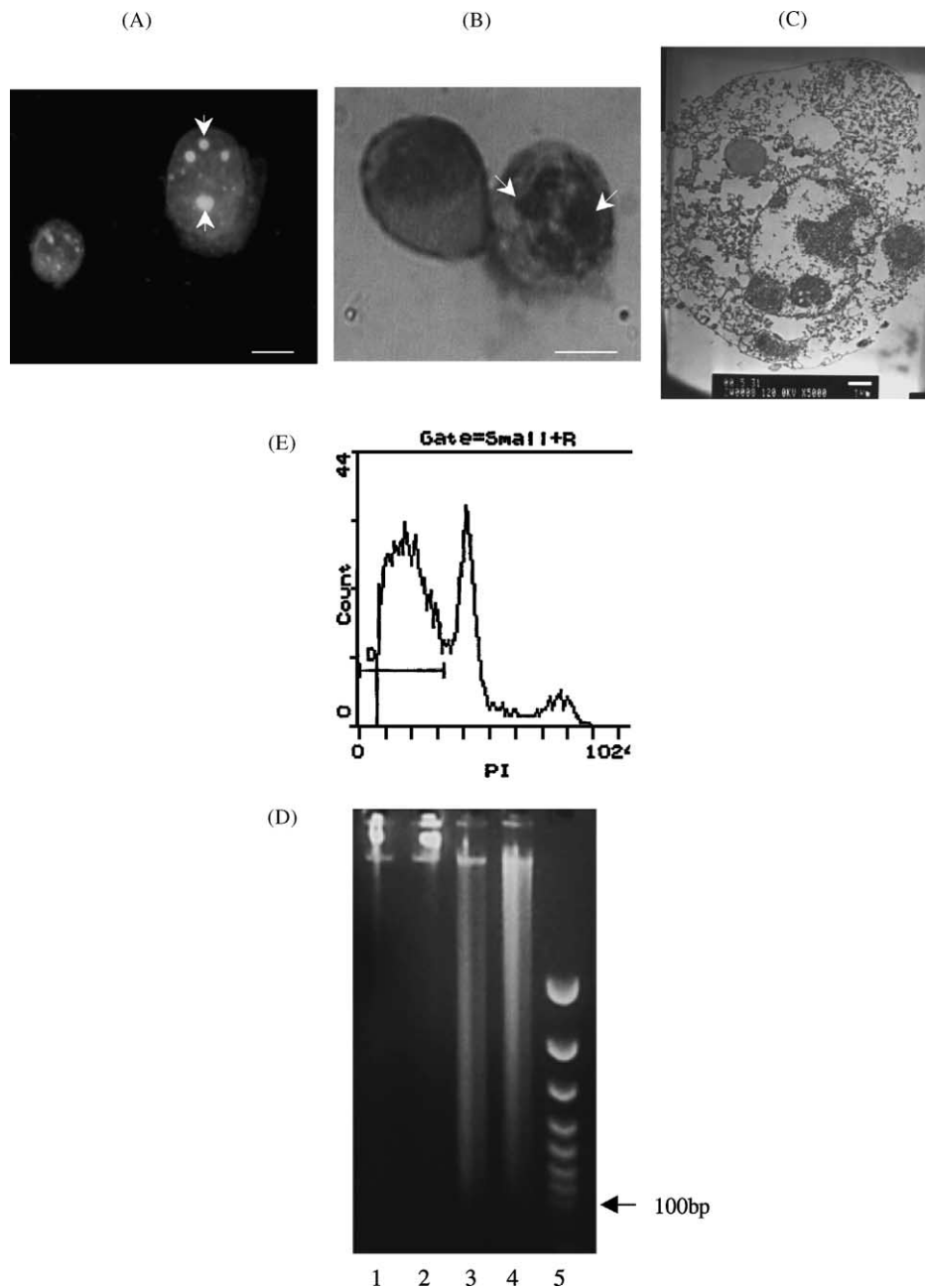


Fig. 6. Morphological, biochemical and flow cytometry features of apoptosis induced by BC-4. HT-1080 human fibrosarcoma cells were treated with BC-4 at 75 μ M for 24 h. The condensed chromatin parts appeared as “apoptotic body” (arrows) were made visible by acridine orange staining (A, bar 10 μ m) and Wright-Giemsa staining (B, bar 10 μ m) and electronmicroscopy (5000 \times , C, bar 1 μ m). DNA laddering (D), lane 1, control; lanes 2–4, cells were treated for 24 h with BC-4 at the concentration of 25, 50, 75 μ M, respectively; lane 5, 100 bp DNA marker. The DNA laddering is a biochemical proof for apoptosis which is further substantiated by PI fluorocytometry showing a sub-G1 peak (E) after treatment with BC-4 at 50 μ M for 36 h, with 59.3% apoptotic cell percent.

demonstrated that at low concentration BC-4 could inhibit the secretion of MMPs. Again, from *in vitro* experiments using electrophoresed enzyme preparations which were co-incubated with BC-4, no direct enzyme inhibition in respect to digest gelatin could be observed (data not shown). This result obtained *in vitro* in a cell-free medium was in line with the results obtained from topoisomerase II results, the enzyme activity of which was also not inhibited

by direct incubation with BC-4. We only see an effect on topoisomerase II and MMPs inhibition when the cells are incubated with BC-4.

We used five experimental settings to prove the idea that BC-4 can induce apoptosis in HT-1080 fibrosarcoma cells. The occurrence of “apoptotic bodies” and the DNA laddering together with the appearance of a sub-G1 peak in flow cytometry analysis strongly suggest that BC-4 is an

apoptosis inducer, the mechanism of which is being investigated.

We have used an array of differential settings to prove the biological effects of BC-4 in tumor cell lines. Taken together the results suggest that BC-4 is able to induce cytostasis, differentiation and apoptosis in the investigated cell lines. We have seen that BC-4 in normal human cells such as human umbilical vein endothelial cells (HUVEC), shows no potential to induce apoptosis. There are ongoing experiments in China to determine the toxicity level of BC-4. If it figures out that there is a low toxicity potential in animal too, then, together with our *in vitro* results, BC-4 is a good candidate being used in chemopreventive intervention strategies, either to interrupt the occurrence of a primary tumor or to decrease the likelihood of metastasis formation.

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Wanzhou Zhao is a stipendiary of the Deutscher Akademischer Austauschdienst (DAAD).