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Naphthoquinones from *Onosma paniculata* Induce Cell-Cycle Arrest and Apoptosis in Melanoma Cells

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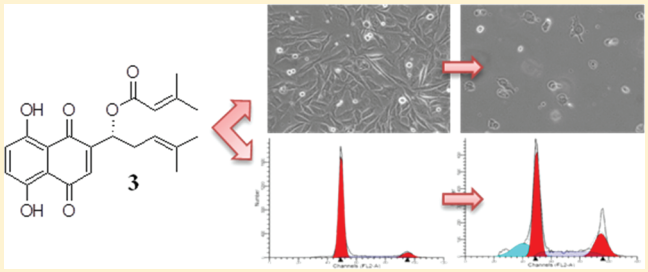
Supporting Information

ABSTRACT: Activity-guided fractionation of a petroleum ether-soluble extract of the roots of *Onosma paniculata*, which has been shown to affect the cell cycle and to induce apoptosis in melanoma cells, led to the isolation of several shikonin derivatives, namely, *β*-hydroxyisovalerylshikonin (**1**), acetylshikonin (**2**), dimethylacrylshikonin (**3**), and a mixture of *α*-methylbutyrylshikonin and isovalerylshikonin (**4**+**5**). All compounds exhibited strong cytotoxicity against eight cancer cell lines and MRC-5 lung fibroblasts, with **3** found to possess the most potent cytotoxicity toward four melanoma cell lines (SBcl2, WM35, WM9, and WM164). Furthermore, **3** and the mixture of **4**+**5** were found to interfere with cell-cycle progression in these cell lines and led to an increasing number of cells in the subG1 region as well as to caspase-3/7 activation, indicating apoptotic cell death.



Onosma paniculata Bureau & Franchet is a biennial herb belonging to the family Boraginaceae. The genus *Onosma* is represented by 145 species centered in Asia, out of which 29 species are found in mainland China.[1] The dried roots of this ("Dian Zi Cao") and related species (e.g., *Arnebia euchroma* ["Ruan Zi Cao"], *Lithospermum erythrorhizon* ["Ying Zi Cao"]) are used in traditional Chinese medicine for the treatment of various diseases including cancer.[2,3] Shikonin and alkannin derivatives are the main constituents and are found in at least 150 species of Boraginaceous genera including *Lithospermum*, *Echium*, and *Onosma*.[4] Their pharmacological activities range from wound-healing to anti-inflammatory, anticancer, and antimicrobial and antiviral properties.[5−16] However, *O. paniculata* is presently little investigated regarding its constituents and pharmacological activities. In a previous study by our group, it was shown that a petroleum ether extract of the roots exhibited strong growth inhibitory activity toward cancer cell lines and induced cell death caspase-dependently.[17] We now have undertaken bioassay-guided fractionation and report the isolation and structure elucidation of the active principles and their effects on cancer cell lines.

RESULTS AND DISCUSSION

HPLC analysis of the active petroleum ether-soluble extract pointed to several shikonin derivatives.[17] Using preparative HPLC, seven fractions were collected, from which fractions 2, 4, 6, and 7 gave *β*-hydroxyisovalerylshikonin (**1**), acetylshikonin (**2**), dimethylacrylshikonin (**3**), and a mixture of *α*-methylbutyrylshikonin (**4**) and isovalerylshikonin (**5**), respectively.

Since it has been demonstrated that biological activity can be lost due to polymerization during isolation processes,[4] the one-step isolation procedure used was a great benefit in the present work. The isolates were identified using ^{1}H and ^{13}C NMR and CD spectroscopic measurements (Tables S1 and S2, Supporting Information) and by comparison with published data.[12,18] Compounds **4**+**5** were obtained as a mixture and could not be separated, but ^{1}H and ^{13}C NMR data clearly revealed them to occur in a ratio of 2:1. The enantiomers shikonin and alkannin give identical NMR spectra[19] but vary in configuration at C-11. They are not capable of being differentiated by optical rotation with a sodium lamp as light source, but CD measurements may be used for this purpose. All

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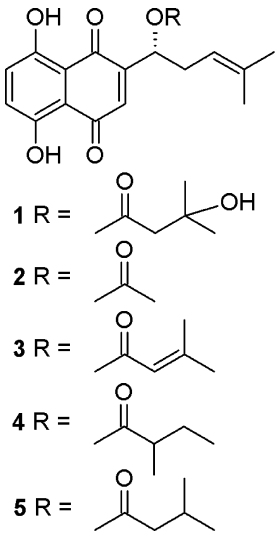

isolated compounds exhibited a positive Cotton effect and were identified as shikonin derivatives.[19,20] This is the first report concerning the CD data of **1**−**3**.

Subsequently, the compounds were evaluated for their cytotoxic potential using eight human cancer cell lines and human nontumorigenic MRC-5 lung fibroblasts. Cytotoxicity was compared to vinblastine, which was used as positive control (Table 1). All isolated compounds exhibited cytotoxicity with IC_{50} values ranging from 600 nM to 70 μM. The most sensitive was the CCRF-CEM leukemia cell line, showing up to 6-fold lower IC_{50} values than MRC-5 cells. The four melanoma cell lines (SBcl2, WM35, WM9, and WM164) were isolated primarily from different stages of melanoma progression.[21] It has been shown that numerous genetic mutations leading to altered cell signaling are involved in the transformation from benign nevi to melanoma.[22] These changes enable melanoma cells to escape apoptosis and contribute to unrestricted cell proliferation. The effects of **3** and the mixture of **4**+**5** regarding cell morphology, cell-cycle distribution, and apoptosis induction were analyzed. Test compounds were freshly dissolved in DMSO and used immediately since storage in DMSO led to a considerable loss of activity. Compound **3** exhibited the lowest IC_{50} values toward melanoma cell lines, which is probably due to the presence of an unsaturated carbonyl group in the side chain. Morphological changes (Figure S3, Supporting Information) were observed after 24 h and continued up to 48 h. Cells exposed to the IC_{50} concentrations of **3** or **4**+**5** exhibited less density and a more round shape phenotype with fewer dendrites. It has been reported that shikonin induces apoptosis in several cell lines[23−25] and affects the cell cycle.[24,26] However, compound **3** and **4**+**5** are less well investigated. Investigations of the cell-cycle distribution revealed that **3** and **4**+**5** strongly affected the amount of cells in the G1 phase and, accordingly, the G2/M or S phase. For **3**, it was found that there were up to 50% fewer cells in the G1 phase and an increasing number of cells in the S phase (SBcl2 and WM35) or G2/M phase (WM9 and WM164) (Figure 1). In contrast to **3** and also **4**+**5**, shikonin led to an arrest in the G1 phase in melanoma[26] and bladder[24] cancer cells, while cells in the G2/M phase decreased, indicating a different mode of action. Additionally, cells in the subG1 region (up to about 20%) increased after 24 h (up to ca. 20%), indicating apoptotic cell death, which was confirmed using a CaspaseGlo 3/7 assay (Figure 2). The results for cells

Table 1. IC_{50} (μM) Concentrations of Isolated Compounds after 72 h of Treatment[a]

	tumorigenic cell line								nontumorigenic cell line
compound	CCRF-CEM	MDA-MB-231	U251	HCT 116	SBcl2	WM35	WM9	WM164	MRC-5
1	0.6 ± 0.1	4.6 ± 0.3	23.6 ± 0.2	4.1 ± 0.6	8.3 ± 0.3	13.0 ± 2.1	12.7 ± 0.4	30.9 ± 1.4	3.9 ± 0.1
2	1.0 ± 0.1	11.3 ± 1.7	15.9 ± 0.7	9.0 ± 0.5	13.0 ± 0.7	30.7 ± 1.1	23.5 ± 0.7	71.5 ± 1.9	2.6 ± 0.2
3	1.9 ± 0.3	23.2 ± 0.4	30.8 ± 1.1	20.0 ± 0.8	1.1 ± 0.1	2.3 ± 0.1	2.7 ± 0.3	8.3 ± 0.3	2.4 ± 0.4
4 +**5**[b]	1.7 ± 0.1	14.7 ± 1.7	22.7 ± 1.4	1.3 ± 0.1	15.5 ± 0.4	23.0 ± 1.8	18.8 ± 0.5	52.3 ± 0.8	3.6 ± 0.2
vinblastine	$9.4 \times 10^{-3} \pm 2.0 \times 10^{-4}$	$3.1 \times 10^{-2} \pm 4.6 \times 10^{-3}$	$8.1 \times 10^{-3} \pm 1.0 \times 10^{-3}$	$8.7 \times 10^{-3} \pm 5.0 \times 10^{-4}$	$1.1 \times 10^{-2} \pm 1.6 \times 10^{-3}$	$3.5 \times 10^{-3} \pm 6.0 \times 10^{-4}$	$1.5 \times 10^{-2} \pm 2.4 \times 10^{-3}$	$4.2 \times 10^{-3} \pm 1.3 \times 10^{-3}$	$2.4 \times 10^{-2} \pm 3.3 \times 10^{-3}$

[a]Results are expressed as means ± SEM of six independent experiments. IC_{50} values were determined using the four-parameter logistic curve and individual values of all independent experiments. [b]Tested as an inseparable 2:1 mixture of **4**+**5**.

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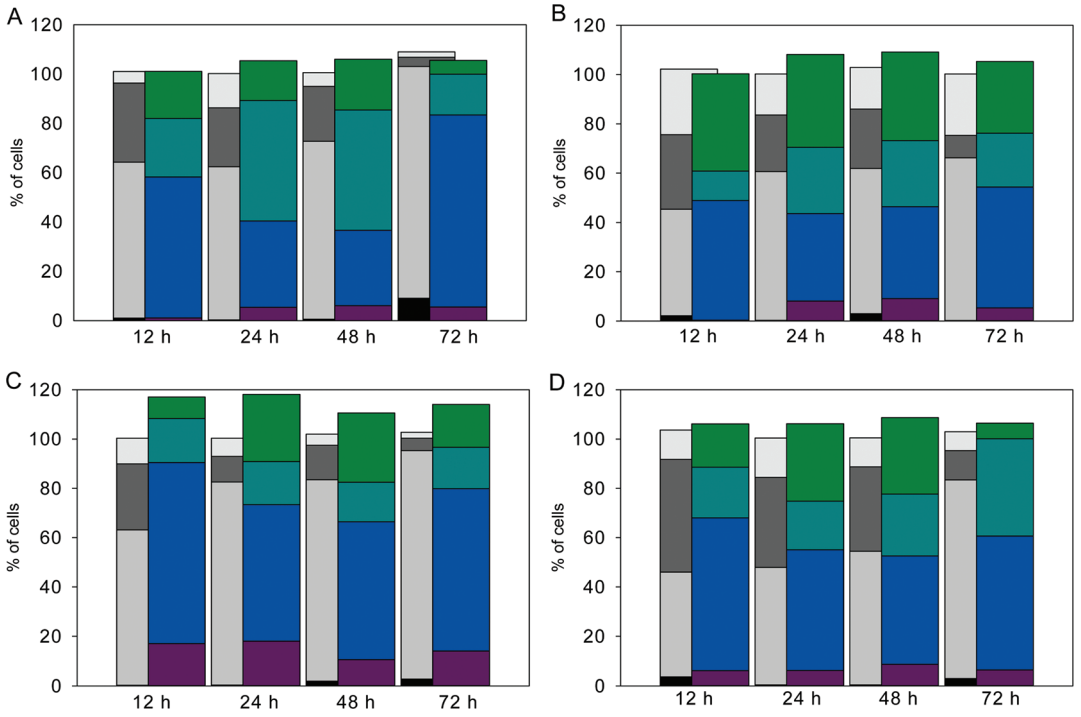

Figure 1. Treatment of melanoma cells with **3** reduced cells in the G1 phase and increased cells especially in the S-phase (SBcl2 (A) and WM35 (B)), and G2/M phase (WM9 (C) and WM164 (D)). Gray bars: Vehicle-treated control cells. Colored bars: Cells exposed to **3** (IC_{50}). The times shown represent the duration of treatment, $n = 3$, with mean values shown. Areas from the bottom to the top: subG1 cells (black and purple), G1 phase cells (lower light gray and blue), S phase cells (dark gray and turquoise), and G2/M phase cells (upper light gray and green).

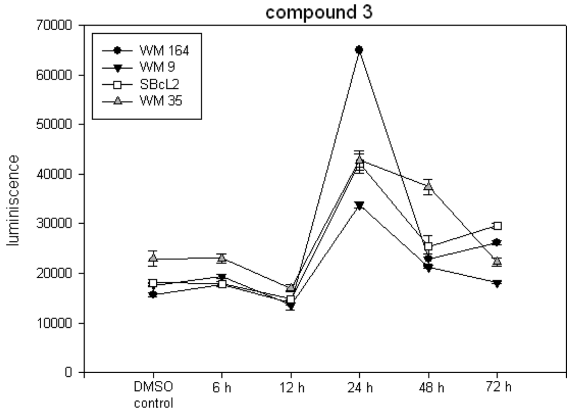

Figure 2. Caspase 3/7 activity measured using the Caspase-Glo 3/7 assay. Caspase activity peaked after 24 h when cells were exposed to the IC_{50} value of **3**, indicating apoptotic cell death.

exposed to the IC_{50}'s of **4**+**5** were similar (Figures S3−S5, Supporting Information).

In summary, it has been shown that roots of *O. paniculata* are a rich source of acetylshikonin (**2**), dimethylacrylshikonin (**3**), α-methylbutyrylshikonin (**4**), and isovalerylshikonin (**5**). All of these compounds exhibited cytotoxicity toward a panel of cancer cell lines. For the melanoma cell lines used, the results indicated that **3** and the mixture of **4**+**5** affect the cell cycle and induce apoptosis.

EXPERIMENTAL SECTION

General Experimental Procedures. CD measurements were recorded on a JASCO J-715 spectropolarimeter, at 210−600 nm in $CHCl_3$. NMR spectra were recorded in chloroform-d_1 (Eurisotop, Saint-Aubin Cedex, France) on a Varian Unitylnova 400 MHz (400 MHz for ^1H and 100 MHz for ^{13}C) or 600 MHz (600 MHz for ^1H and 150 MHz for ^{13}C) spectrometer at 25 °C using TMS as internal standard. For analytical HPLC experiments, a LiChroCART RP18 column (250 × 4 mm, 5 μm) and a Merck Hitachi system consisting of an L-7100 pump, L-7200 autosampler, L-7455 diode array detector, and a D-7000 interface were used. Preparative reversed-phase HPLC was performed on a Varian R PrepStar SD-1 with Dynamax R solvent delivery system and an absorbance detector model UV-1 using a VDSpher 100 RP18 column (250 × 25 mm, 10 μm). For both, the mobile phase consisted of water (A) and ACN (B). The following gradient was used: 0−45 min, 70−100% B; 45−60 min, 100% B.

Plant Material. The dried roots of *O. paniculata* were purchased at a medicinal plant market in Kunming, China, in November 2003. They were identified macroscopically by Prof. Xiao-Jiang Hao, and the identity was confirmed based on its ITS and trnL-F regions by Prof. Guenther Heubl as described previously.[17] A voucher specimen is deposited at the herbarium of the Institute for Plant Sciences, University of Graz, Austria.

Extraction and Isolation. The petroleum ether extract was prepared by Soxhlet extraction.[17] A 400 mg aliquot of this extract was dissolved in MeOH (40 mg/mL) and fractionated using preparative HPLC. Seven fractions were collected, from which fractions 2, 4, 6, and 7 gave **1** (1.1 mg), **2** (31.1 mg), **3** (48.6 mg), and **4**+**5** (46.3 mg), respectively.

*β-Hydroxyisovalerylshikonin (**1**):* red powder; CD (*c* 2.5 mM, $CHCl_3$) $[\theta]_{315}$ −15 450, $[\theta]_{365}$ +15 910; ^1H NMR ($CDCl_3$, 400 MHz) data, see Table S1 Supporting Information; ^{13}C NMR ($CDCl_3$, 100 MHz) data, see Table S2 Supporting Information.

*Acetylshikonin (**2**):* red powder; CD (*c* 5.0 mM, $CHCl_3$) $[\theta]_{305}$ −1470, $[\theta]_{356}$ +1650; ^1H NMR ($CDCl_3$, 600 MHz) data, see Table S1 Supporting Information; ^{13}C NMR ($CDCl_3$, 150 MHz) data, see Table S2 Supporting Information.

*Dimethylacrylshikonin (**3**):* red powder; CD (*c* 4.0 mM, $CHCl_3$) $[\theta]_{315}$ −990, $[\theta]_{359}$ +540; ^1H NMR ($CDCl_3$, 400 MHz) data, see Table S1 Supporting Information.

*α-Methylbutyrylshikonin (**4**) and Isovalerylshikonin (**5**):* red powder; ^1H NMR ($CDCl_3$, 600 MHz) data, see Table S1 Supporting

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Information; ^{13}C NMR ($CDCl_3$, 150 MHz) data, see Table S2 Supporting Information.

Sample Preparation. The compounds were freshly dissolved in DMSO, subsequently diluted with medium, and used immediately. Control cells represent vehicle-treated cells (0.5% DMSO). The final DMSO concentration did not affect the cells.

Cell Culture. Human CCRF-CEM leukemia and MDA-MB-231 breast cancer cells lines were maintained in RPMI 1640 medium (Sigma-Aldrich, St. Louis, MO, USA), supplemented with 2 mM L-glutamine (Sigma-Aldrich), 10% fetal bovine serum (FBS, PAA Laboratories, Pasching, Austria), 100 units/mL penicillin (PAA), and 100 μg/mL streptomycin (PAA) (1% penicillin/streptomycin). Human U251 glioblastoma and HCT 116 colon cancer cell lines were grown in high-glucose Dulbecco's modified Eagle medium (DMEM, Sigma-Aldrich) supplemented with 2 mM L-glutamine, 10% FBS, and 1% penicillin/streptomycin. Human SBcl2, WM35, WM9, and WM164 melanoma cell lines were grown in RPMI 1640 medium with 2 mM L-glutamine, 2% FBS, and 1% penicillin/streptomycin. Human MRC-5 lung fibroblasts were grown in minimum essential medium (MEM, Gibco, Invitrogen, Vienna, Austria) supplemented with 2 mM L-glutamine, 10% FBS, and 1% penicillin/streptomycin. All cells were kept in a humidified 5% CO_2 atmosphere at 37 °C and passaged at 90% confluence. The cell cultures were periodically checked for the presence of mycoplasma by PCR.

XTT Viability Assay. The XTT viability assay was performed as described previously[17] and in accordance with the manufacturer's protocol (Roche Diagnostics, Mannheim, Germany; cell proliferation kit II (XTT), cat. no 11 465 015 001). In brief, 10 000 cells/well in the case of CCRF-CEM and MRC-5 cells and 5000 cells/wells for the other cell lines were seeded into 96-well plates (100 μL, flat bottom) and treated with various concentrations of **1**−**3** or **4**+**5** for 72 h. Adherent cell lines were grown overnight before the test compounds were added. After 72 h, a freshly prepared XTT solution (5 mL of XTT plus 100 μL of electron coupling reagent) was added and analyzed after another 1.5 or 4 h (CCRF-CEM cells) using a Victor^2 1420 multilabel counter (PerkinElmer Life Sciences, Waltham, MA, USA). Vinblastine served as positive control (0.01 μM).

Cell-Cycle Analysis. Cells were treated with the respective IC_{50} concentration for 12−72 h and harvested by trypsinization. Then, 5×10^5 cells were fixed with 70% ice-cold EtOH for 10 min at 4 °C. After washing with PBS, the cell pellet was resuspended in propidium iodide (PI) staining buffer (50 μL/mL PI, RNase A, Beckman Coulter, Krefeld, Germany) and incubated at 37 °C for 15 min. Cell-cycle distribution was analyzed using a FACSCalibur flow cytometer (BD Biosciences, Heidelberg, Germany) using Modfit Software version 3.0 (Verity Software House, Topsham, ME, USA).

Caspase-Glo 3/7Assay. For this assay, 10 000 cells/well (100 μL) were treated with the respective test-compound IC_{50} concentration for 6−72 h and analyzed for caspase activation using the Caspase-Glo 3/7 assay (Promega Corporation, Madison, WI, USA), according to the manufacturer's protocol. Luminescence was measured 30 min after adding the Caspase-Glo 3/7 reagent (Caspase-Glo substrate and buffer).

Statistical Analysis. Statistical analysis was carried out using SigmaPlot 12.0 (Systat Software Inc., Chicago, IL, USA). IC_{50} values were determined using the four-parameter logistic curve, at least eight concentrations of the test compound, and two different cell passages each tested in three independent wells. Results are expressed as mean ± SEM.

■ ASSOCIATED CONTENT

Supporting Information

^1H and ^{13}C NMR spectroscopic data of **1**−**5**, photographs of morphological changes (24 and 48 h) caused by **3** and **4**+**5**, as well as the effects of compounds **4** +**5** on the cell-cycle (12−72 h) distribution and activation of caspase 3/7 (6−72 h). This information is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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