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LABORATORY INVESTIGATION

Curcumin acts anti-proliferative and pro-apoptotic in human meningiomas

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Abstract Meningiomas, the most frequent benign intracranial and intraspinal types of tumors are normally removed by surgery. Complications can occur when the tumor is critically localized and cannot be completely removed or when comorbidities of the mostly elder patients increase the general surgical risk. Thus, alternate medical treatment concepts for the therapy of meningiomas would be desirable. Curcumin, the active ingredient of the spice plant *Curcuma longa* has shown anti-tumorigenic actions in many different types of tumors and therefore, its effect on growth and apoptosis of meningioma cells was studied in the present paper. In vitro, treatment of the human Ben-Men-1 meningioma cell line and of a series of 21 primary human meningioma cell cultures with curcumin (1–20 μM) strongly reduced the proliferation in all cases in a dose dependent manner. Cell cycle analysis by fluorescence-activated cell sorting showed growth arrest at G2/M phase, which was confirmed by demonstrating the corresponding modulation of proteins involved in G2/M arrest by immunoblotting and/or confocal laser microscopy. High dosages (20, 50 μM) of curcumin induced a significant increase of apoptosis in Ben-Men-1 and primary meningioma cell cultures as demonstrated by morphological changes of cell nuclei, DNA fragmentation, translocation of cell membrane associated phosphatidyl serine and the induction of apoptotic-acting cleaved caspase-3. Our results suggest that the multi-targeting drug curcumin has potent anti-tumorigenic actions in meningioma cells and might therefore be a putative candidate for the pharmacological treatment of meningiomas.

Keywords Meningioma · Curcumin · Proliferation · Apoptosis · G2/M phase cell cycle arrest

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Introduction

With a proportion of 20–25 %, meningiomas represent the most abundant type of primary intracranial and spinal tumors [1–3]. They derive from morphologically and functionally diverse cells of the meninges and thus form histologically different subtypes [1, 4]. Based on the degree of aggressiveness, meningiomas are classified by the World Health Organisation (WHO) as benign WHO grade I (more than 80 % of all meningiomas), more aggressive grade II (15–20 %) and malignant grade III (less than 3 %) tumors [4].

Regarding the pathogenesis of meningiomas, the most frequent genetic alteration observed is the inactivation of

the neurofibromatosis 2 (NF2) gene encoding the tumor suppressor merlin [5]. Other, less frequent mutations affect the genes for DAL1 and as recently described, SMARCE1, TRAF7, KLF4, AKT1 and SMO [6–8]. The combination of some of these mutations seems to be associated with enhanced malignancy [9]. Changes in the expression of tumor suppressors, hormone receptors (e.g. progesterone receptor; somatostatin receptors), growth factors and angiogenic factors (e.g. PDGF, EGF, TGF-α, TGF-β, endothelin-1, VEGF) as well as their receptors and subsequent signaling proteins are also thought to play a significant role in meningioma development [1, 2, 10–12].

Surgical resection is the primary therapy of meningiomas and is successful in most patients. However, critically localized and thus incompletely resected meningiomas regrow and an increased recurrence rate has been reported for more aggressive meningioma types. Additional resection and/or radiotherapy are then applied to control recurrent meningiomas as so far no effective pharmacological therapy of theses tumors is available [2, 3, 13, 14].

Curcumin, the active ingredient of the spice plant *Curcuma longa* has potent anti-tumorigenic actions in many different types of tumors by suppressing proliferation and angiogenesis and/or supporting apoptosis [15, 16]. The multi-targeting drug curcumin affects aberrantly expressed growth factors/receptors, tumor suppressors (among them merlin), oncogenes and over-activated intracellular signaling components [15–18] some of which are disturbed in meningiomas. Therefore, we have studied in the present paper for the first time whether curcumin has anti-proliferative and pro-apoptotic effects on meningioma cells.

Materials and methods

Materials

Cell culture materials and reagents were obtained from Life Technologies (Karlsruhe, Germany), Falcon (Heidelberg, Germany), Nunc (Wiesbaden, Germany), Seromed (Berlin, Germany), Flow Cytometry Standards Corp. (Meckenheim, Germany) and Sigma. Curcumin (95 %, turmeric powder) and dimethylsulphoxide (DMSO) were purchased from Sigma (St. Louis, USA).

Cell culture and curcumin stimulation

Human Benign-Meningioma-1 (Ben-Men-1) cells were cultured at 5 % CO2 and 37 °C in DMEM (with 4.5 g/l D-glucose, pH 7.3) supplemented with 10 % FCS, 2 mM glutamine, 2.5 mg/l amphotericin B and 10^5 U/l penicillin–streptomycin.

Table 1 Clinical and histological characteristics of the meningiomas studied

Tumor	sex/age	Histological type	WHO-grade	P-index (%)[a]
M1	f/74	Meningothelial	I	7
M2	m/73	Meningothelial	I	<3
M3	f/54	Transitional	I	2
M4	f/62	Atypical	II	10
M5	f/37	Fibroblastic	I	1
M6	m/54	Meningothelial	I	1
M7	m/56	Fibroblastic	I	10
M8	m/58	Meningothelial	I	nd
M9	f/50	Meningothelial	I	<5
M10	f/60	Meningothelial	I	10
M11	f/49	Microcystic	I	1
M12	m/71	Meningothelial	I	6
M13	m/65	Meningothelial	I	1
M14	m/73	Fibroblastic	I	5
M15	f/80	Atypical	II	10
M16	f/72	Meningothelial	I	<2
M17	f/74	Meningothelial	I	3
M18	m/71	Psammomatous	I	<2
M19	m/77	Meningothelial	I	6
M20	m/71	Meningothelial	I	5
M21	f/57	Meningothelial	I	4

[a] Proportion of Ki-67 expressing cells determined by MIB-1 immunostaining. *f* female, *m* male, *P* proliferation, *WHO* World Health Organisation, *nd* not done

For primary human meningioma cell culture, tumor tissue was obtained by transcranial surgery from 21 patients with various types of meningiomas as indicated in Table 1. None of the patients had neurofibromatosis type 2 (NF2), a hereditary disease associated with meningioma formation. With the exception of 2 atypical WHO-grade II meningiomas only benign tumors, and in most cases meningothelial meningiomas were studied. Dispersion of the tumor tissues was performed in the same way as previously described [19] and the meningioma cells were cultured in the same medium as used for Ben-Men-1 cells. Due to the limited amount of human meningioma tissue available, not all experiments could be done in parallel in all primary human meningioma tumor cell cultures.

The present work was approved by the local ethics committee, and written informed consent was obtained from all patients included in the study.

For in vitro experiments, a stock solution of 10 mM curcumin (prepared in DMSO) was diluted with cell culture medium to obtain concentrations of 1–20 μM (in some cases up to 50 μM) curcumin for cell treatments. During curcumin application for various time periods as indicated, light exposure was reduced as much as possible. Vehicle control studies with medium containing 0.2 or 0.5 %

DMSO corresponding to the amount present in the highest curcumin dosages of 20 or 50 μM were performed in each experiment to exclude effects of DMSO.

In vitro proliferation assays

To study the effects of curcumin on the proliferation of Ben-Men-1 cells and primary human meningioma cell cultures, ^{3}H-thymidine incorporation experiments were performed as previously described [19]. For Ben-Men-1 cells, the results obtained by ^{3}H-thymidine incorporation were confirmed by direct cell counting. To this end, Ben-Men-1 cells (30,000 cells per well) were seeded into a 48 well-plate. After an over-night attachment period the number of cells attached were counted in some wells and then the Ben-Men-1 cells were treated for different time periods with increasing concentrations of curcumin as indicated. Subsequently the cells were removed from the wells by trypsin/EDTA treatment and counted with a cell size-adapted coulter counter (Beckman-Coulter, Krefeld, Germany) according to the instructions of the manufacturer.

Fluorescence-activated cell sorting analysis

Fluorescence-activated cell sorting (FACS) analysis was performed to determine the effect of curcumin on cell cycle arrest of human meningioma cells. Cells were treated for 24 h, harvested by trypsinization and fixed in 70 % ethanol at 4 °C overnight. Samples of fixed cells were resuspended and stained in PBS containing 20 μg/ml Propidium Iodide (PI) and 10 μg/ml RNAse A. FACS analysis of PI emission at 630 nm (Fl4) was performed using a Beckman Coulter (Krefeld, Germany) XL flow cytometer, and cell cycle analysis of DNA histograms was done using the Multicycle software (Phoenix Flow Systems, San Diego, CA, USA).

Apoptosis/necrosis was also assessed by FACS analysis in Ben-Men-1 cells after treatment with 10–20 μM curcumin using Annexin V-FITC (Beckman Coulter, Krefeld, Germany) with a commercially available kit (Beckman Coulter, IM3614). Apoptotic cells were defined as cells that stained positive for Annexin V-FITC binding to phosphatidyl serine residues on outer cell membranes, whereas 7-aminoactinomycin D (7-AAD)-positive cells were considered to be necrotic. To each tube, 20 μl 7-AAD, 10 μl Annexin V-FITC and 100 μl 1 × Binding Buffer (Beckman Coulter) were added and incubated on ice for 15 min in the dark. Post incubation, 300 μl 1 × Binding Buffer (Beckman Coulter) was applied to each tube. Excitation was at 488 nm, the FITC fluorescence was recorded on a fluorescence 1 (FL1) sensor (515–545 nm-BP), and the 7-AAD fluorescence was recorded on a FL4 sensor (600 nm-LP). Data analysis was performed with Coulter XL software EXPO 32.

Western blot analysis

To study the effects of curcumin on various cell cycle regulating proteins, Ben-Men-1 and primary human meningioma cells were treated with DMSO and curcumin as indicated, and after protein extraction, western immunoblotting was performed as previously described [20]. Primary antibodies for the detection of cyclin B1, cyclin E, cyclin D1, CDK-1, CDK2, p-CDK1-Thr15 (all from Cell Signalling Tech., Beverly, MA, USA) and CDK4 (Santa Cruz Biotech., Santa Cruz, CA, USA) were made in rabbit, and anti-β-actin (Millipore Corp., Billerica, MA, USA), anti-p21 (BD biosciences) antibodies were made in mouse. HRP-conjugated secondary antibodies against rabbit and mouse were all obtained from Cell Signalling Tech. Western blot bands were quantified by densitometry with Image J software (NIH, Bethesda, MD, USA), normalized to their correspondent β-actin signal and compared with DMSO-treated controls.

Immunofluorescence assay

One day before treatment, cells were cultured onto Falcon culture slides (BD Biosciences). Ben-Men-1 and primary human meningioma cells were stimulated with the indicated concentrations of curcumin for 24 h. After treatment, cells were fixed in 4 % paraformaldehyde for 5 min on ice and then blocked in 5 % goat serum with 0.1 % (v/v) triton ×100 for 1 h at room temperature. Slides were incubated with rabbit anti-Cyclin B1 (1:50, cell signaling), rabbit anti-CDK1 (1:50, cell signaling), rabbit anti-p-CDK1-Thr15 (1:50, cell signaling), mouse monoclonal anti-p21 (1:50, BD biosciences) and rabbit Cleaved Caspase-3 (Asp175) (1:400, Cell Signaling, Danvers MA, USA) overnight at 4 °C, and then washed and incubated with Alexa Fluor® 594 goat anti-rabbit antibody (1:500, Invitrogen) or FITC-488 goat anti-mouse (1:500, Invitrogen) antibody at room temperature for 2 h. After washing with PBS, ProLong® Gold antifade reagent with DAPI (Invitrogen) was used for mounting and visualization of cell nuclei. Images were obtained using a confocal microscope (Fluo View™ FV1000, Olympus, Munich, Germany). Images were obtained using 20× or 60× objectives.

Statistical analysis

Each of the experiments with Ben-Men-1 cells was repeated at least three times. The individual experiments were performed with quadruplicate wells. Results were analyzed by one-factorial univariate ANOVA and followed by

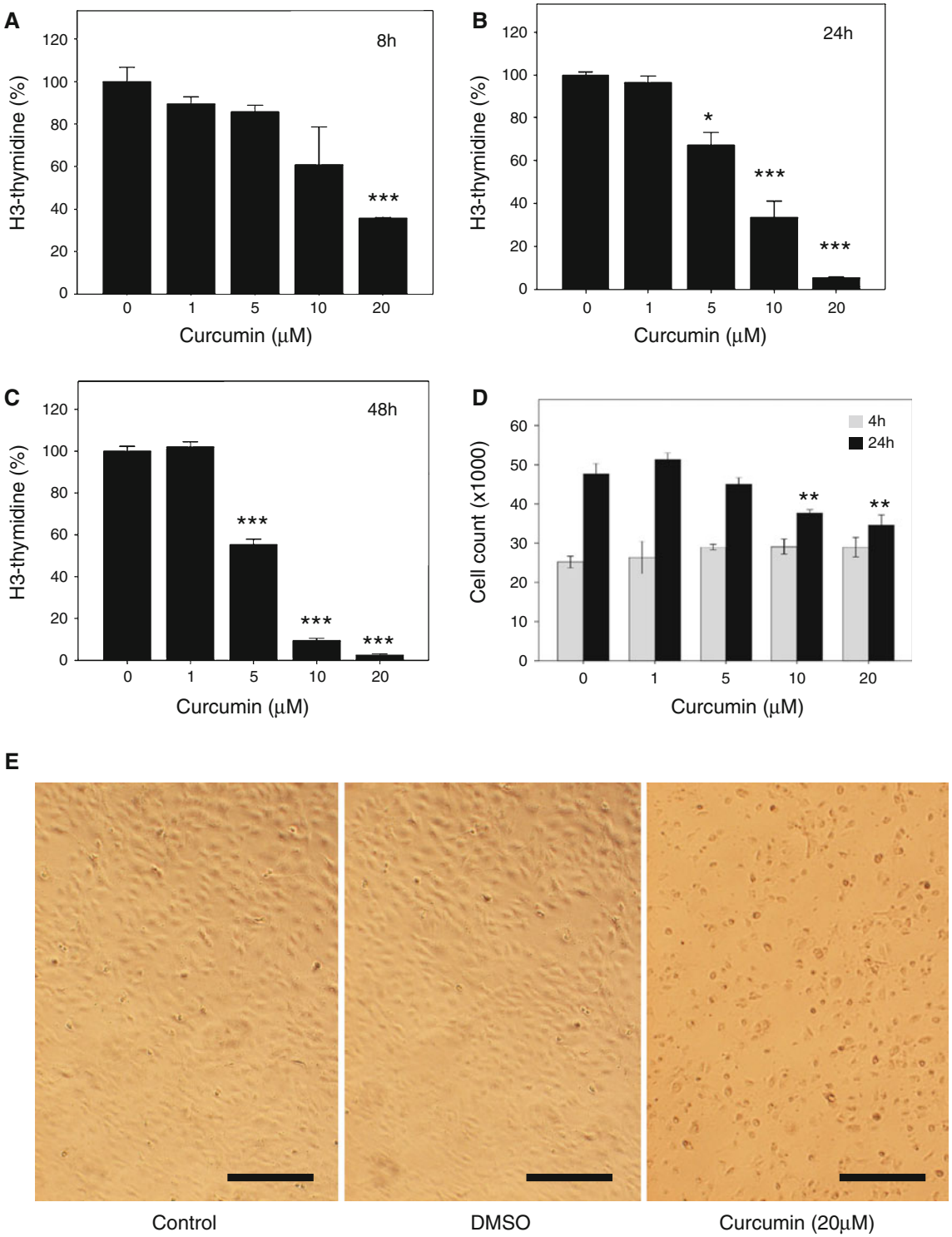


Fig. 1 Effect of curcumin on growth of human meningioma cells. Ben-Men-1 cells were treated with 1–20 μM curcumin and effects on ^{3}H-thymidine incorporation and cell numbers were determined. Representative figures show the dose-dependent inhibition of ^{3}H-thymidine incorporation in Ben-Men-1 cells by curcumin after treatment for 8 h (**a**), 24 h (**b**) and 48 h (**c**). In figure **d** is shown that curcumin has no short-term effect (4 h treatment, *grey bars*) on Ben-Men-1 cell counts but dose-dependently and significantly reduced them after treatment for 24 h (*black bars*). In control experiments, treatment with DMSO (0.2 %) had no significant influence on ^{3}H-tymidine incorporation or cell numbers (not shown). In figure **e** also no difference between untreated and DMSO-treated cells was microscopically seen whereas after curcumin application for 24 h a reduction of cell numbers and moreover a change in the morphology of Ben-Men-1 cells could be observed. The *scale bars* in **e** correspond to 100 μM. All data represent mean ± SEM of quadruplicates. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ versus non-treated cells

univariate F tests where appropriate. As nominal level of significance, $p = 0.05$ was accepted; it was corrected (according to Bonferroni correction procedure) for all post hoc tests, in order to keep the type I error ≤ 0.05. Data are given as mean ± SEM.

Results

Effect of curcumin on meningioma cell proliferation

In two in vitro proliferation assays, ^{3}H-thymidine incorporation and determination of cell number, curcumin dose and time dependently inhibited growth of Ben-Men-1 cells (Fig. 1a–d). After 24 and 48 h incubation periods, significant growth inhibition was already achieved after treatment with 5 μM curcumin and maximum effects of curcumin were obtained with 20 μM curcumin. Higher doses (up to 50 μM) of curcumin had no additional significant inhibitory effects on Ben-Men-1 cell growth (not shown). To confirm the anti-proliferative action of curcumin, Ben-Men-1 cell numbers were directly counted after curcumin treatment with a coulter counter. It could be demonstrated that the application of curcumin suppressed the rise in Ben-Men-1 cell numbers during 24 h in dose-dependent manner (Fig. 1d). The reduction of cell numbers, associated with changes of the cell morphology, could also microscopically be seen (Fig. 1e).

Treatment of 21 primary human meningioma cell cultures with concentrations of 1–20 μM curcumin for 24 or in some cases for 48 h significantly and dose-dependently suppressed the proliferation in all cell cultures studied (Table 2). The sensitivity to curcumin was highly variable in primary meningioma cells. In some cultures significant inhibition was already observed at 1 μM curcumin whereas in others only the highest dosage of curcumin significantly suppressed ^{3}H-thymidine incorporation (Table 2). Although atypical meningioma M4 was one of most curcumin-responsive ones, too few atypical tumors could be studied to identify differences in curcumin sensitivity between atypical and benign meningiomas.

Curcumin causes G2/M cell cycle arrest in human meningioma cells

To gain further insight into the mechanism of the growth inhibitiory effect of curcumin, Ben-Men-1 cells and primary cell cultures of 7 human meningiomas were treated with different concentrations of curcumin and analyzed for cell cycle distribution. Results of FACS analysis of Ben-Men-1 cells showed that 24 h treatment with 20 μM curcumin induced an accumulation of cells in the G2/M

Table 2 Effect of curcumin on the proliferation (^{3}H-thymidine incorporation) of primary cell cultures of human meningiomas

Tumor	Curcumin concentration			
	1 μM	5 μM	10 μM	20 μM
M1[a]	75.2 ± 5.9*	65.9 ± 28.0*	59.3 ± 13.2**	9.3 ± 4.0***
M2[a]	nd	101.1 ± 14.3	87.2 ± 18.9	20.5 ± 6.1***
M3[a]	74.3 ± 6.9*	63.4 ± 25.9*	nd	19.1 ± 9.3***
M4[b]	88.9 ± 10.6	36.3 ± 3.2***	14.4 ± 3.5***	8.4 ± 3.9***
M5[a]	102 ± 26.7	105.8 ± 27.6	79.6 ± 12.6	54.3 ± 8.8**
M6[b]	88.2 ± 14.4	82.7 ± 4.6	68.2 ± 9.9*	41.1 ± 2.5***
M7[a]	110.8 ± 22.4	107.5 ± 8.1	44.5 ± 6.7**	31.4 ± 7.5***
M8[a]	105.4 ± 3.6	89.9 ± 12.1	53.5 ± 3.7**	24.0 ± 1.8***
M9[a]	100.2 ± 39.5	102.4 ± 8.5	92.9 ± 5.3	46.4 ± 15.8**
M10[b]	89.3 ± 5.9	84.5 ± 6.4	66.7 ± 1.7**	58.2 ± 4.6**
M11[a]	110.4 ± 21.9	76.1 ± 3.3*	27.0 ± 9.0***	6.5 ± 1.2***
M12[b]	95.8 ± 3.0	84.0 ± 4.7	34.3 ± 3.5***	7.9 ± 1.5***
M13[a]	101.4 ± 2.6	92.6 ± 2.6	60.6 ± 7.0**	7.1 ± 0.8***
M14[a]	108.9 ± 7.9	88.3 ± 7.4	77.4 ± 6.7*	21.3 ± 1.5***
M15[a]	110.0 ± 3.7	104.3 ± 5.2	77.0 ± 5.5**	19.9 ± 3.0***
M16[a]	118.3 ± 39.4	113.1 ± 23.9	87.45 ± 29.1	9.9 ± 2.9***
M17[a]	104.3 ± 12.3	101.8 ± 15.5	77.3 ± 5.5**	14.7 ± 2.8***
M18[a]	111.4 ± 10.35	102.7 ± 2.6	82.8 ± 6.1	10.1 ± 1.3***
M19[a]	103.3 ± 5.1	66.3 ± 4.2*	38.6 ± 5.2**	9.3 ± 0.8***
M20[a]	113.3 ± 13.1	85.2 ± 7.1	55.2 ± 3.9**	26.9 ± 12.0**
M21[a]	89.8 ± 5.7	93.1 ± 17.7	51.9 ± 2.9***	15.5 ± 1.6***

[3] H-thymidine incorporation values (mean ± SEM) of untreated meningioma cells were set to 100 % (basal) and the above listed values represent percent of basal values

Cells of the various meningiomas were treated with curcumin either for 24 ([a]) or 48 ([b]) hours

phase (Fig. 2a, c) accompanied by a corresponding decline of cells in the G1 phase. Curcumin-induced cell cycle arrest in G2/M phase was also observed in all primary meningioma cell cultures studied (in Fig. 2b, c representative results from one human meningioma cell culture are shown).

Curcumin treatment modulates expression of G2/M cell cycle regulatory proteins

To further decipher the molecular mechanism of curcumin-mediated G2/M arrest, western blotting and immunoflorescence were performed to determine the expression of key-signaling proteins of this cell cycle checkpoint in extracts of curcumin-treated cells. In pilot experiments with Ben-Men-1 cells we observed no significant difference in the expression of the cell cycle regulating proteins studied between untreated and DMSO-treated cells (not shown). As in curcumin-treated cells low amounts of DMSO are always present, we used DMSO-treated cells as control in all further western blot and immunofluorescence

Fig. 3 Curcumin treatment modulates expression of G2/M cell cycle regulatory proteins in meningioma cells. **a** Ben-Men-1 cells were treated with DMSO as a control and with increasing dosages of curcumin for 24 h. Cells were harvested and the expression of cyclin B1, cyclin D3, cyclin E, CDK1, p-CDK1-Thy15, CDK2, CDK4, and p21/WAF1/CIP1 were measured in cellular protein extracts by western blot analysis. β-actin antibody was used as a loading control. **b** For immunofluorescence analysis by confocal microscopy Ben-Men-1 cells were treated with curcumin (20 μM) for 24 h. Cells were fixed and then stained with antibodies against cyclin B1, CDK1, p-CDK-1 and p21. Representative confocal microscope images of red-stained target proteins and DAPI-stained (*blue*) cells are shown. *Scale bars* are equal to 10 μm. **c** Primary human meningioma cells were treated with curcumin (0, 5, 20, 50 μM) for 24 h. Cells were harvested and the expression of cyclin B1, CDK1, p-CDK1-Thy15, CDK2, CDK4 and p21/WAF1/CIP1 were measured by western blot analysis. β-actin antibody was used as a loading control. Representative blots from meningioma M12 are shown. **d** Human primary meningioma cells were treated with curcumin (20 μM) for 24 h. Cells were harvested and stained with antibodies against cyclin B1, CDK1, p-CDK-1 and p21. Representative confocal microscope images (here from meningioma M19) of red-stained target proteins and DAPI-stained cells (*blue*) are shown. In 3A and 3C numbers below the western blot lanes correspond to values of densitometric analyses and show the changes versus control (=1). *Scale bars* in B and D are equal to 20 μm

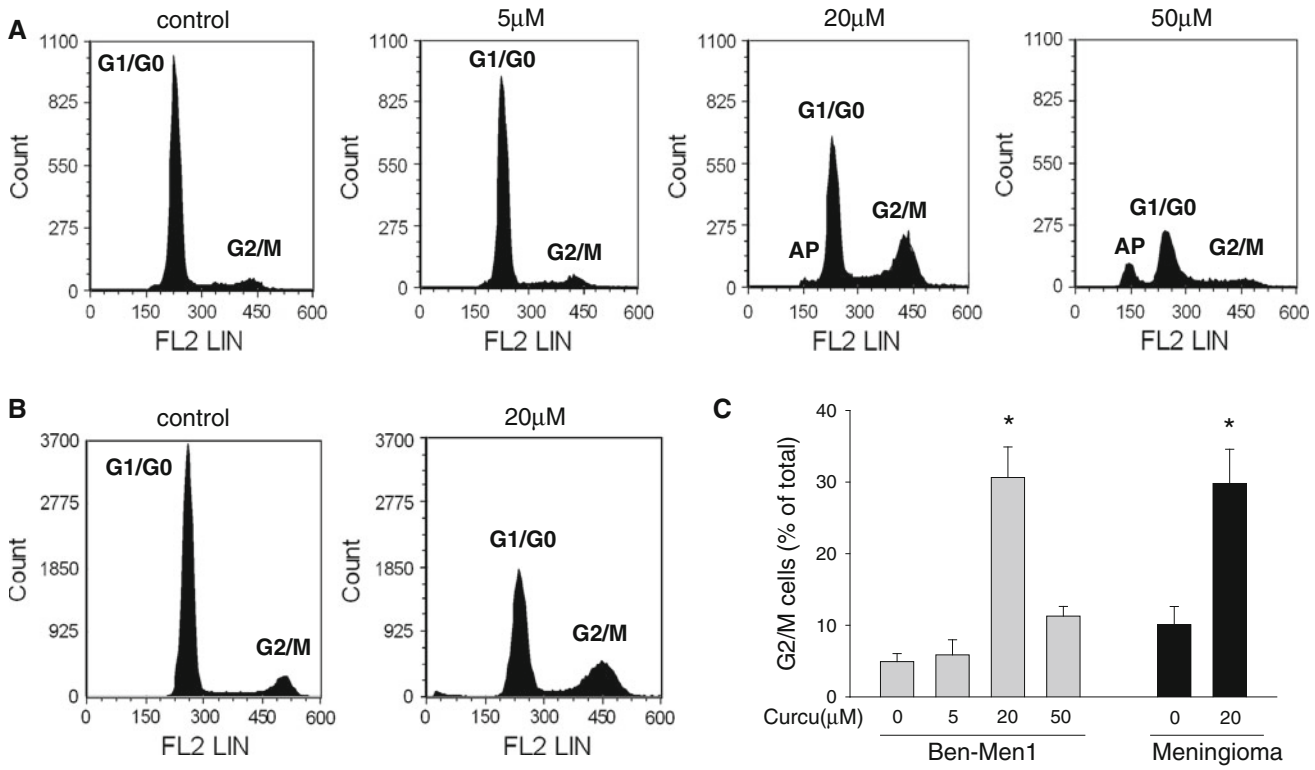
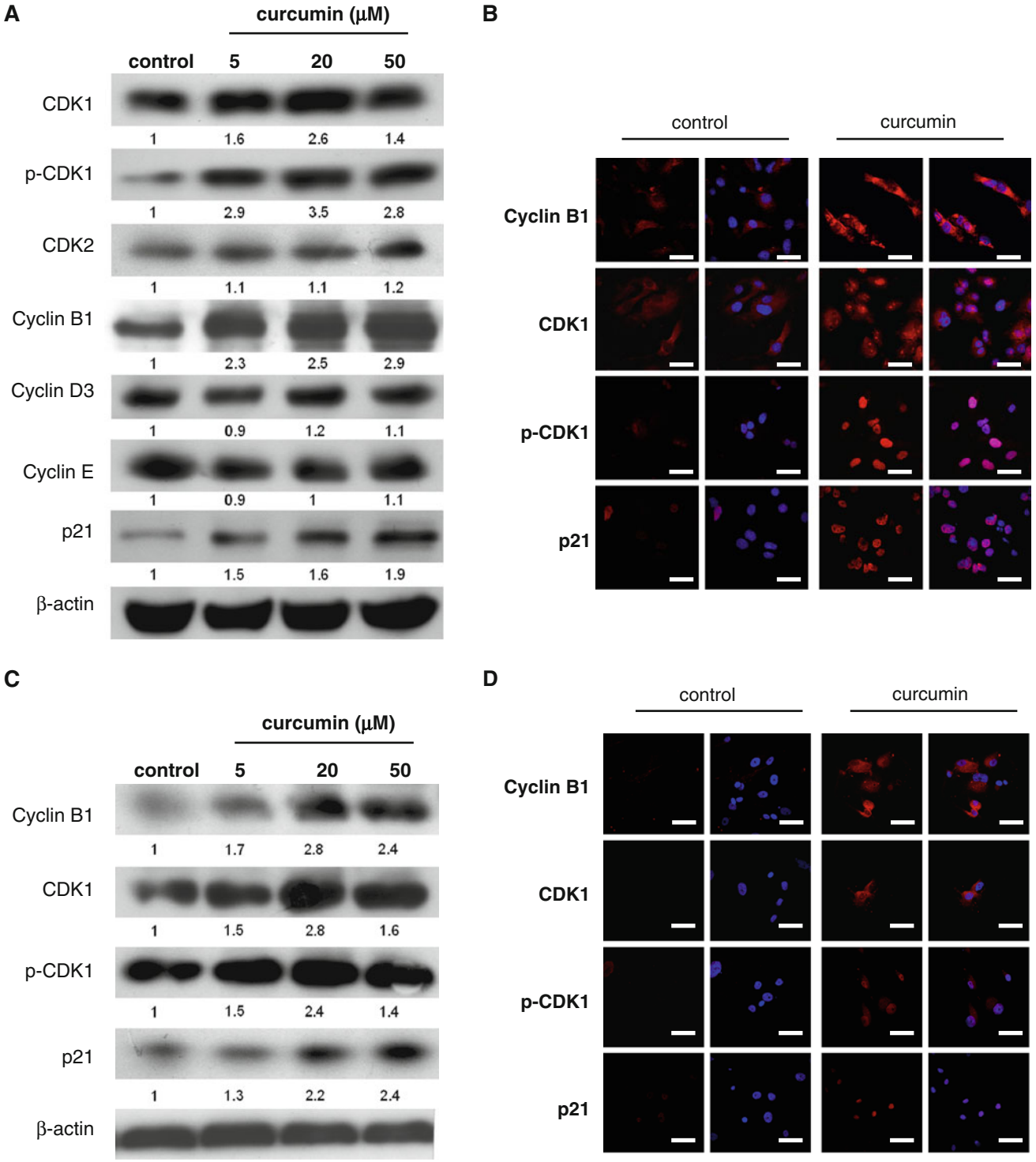


Fig. 2 Curcumin causes G2/M cell cycle arrest in human meningioma tumor cells. **a** Ben-Men-1 cells were treated without or with increasing dosages of curcumin for 24 h. After harvesting, cells were stained with propidium iodide and cell cycle distribution was analyzed by flow cytometry. An increase of cells in G2/M phase (G2/M) was observed reaching its maximum after treatment with 20 μM curcumin (G1/G0: proportion of cells in G1/G0 phase). At higher dosages (20, 50 μM) of curcumin an increase of apoptotic cells (AP) was noted. The histograms presented are representative of at least three separate experiments. **b** Primary human meningioma tumor cells were treated with or without curcumin (20 μM) for 24 h. After harvesting, cells were stained with propidium iodide and cell cycle distribution was analyzed by flow cytometry. A representative histogram of one (meningioma M11) out of 7 human meningiomas studied is shown. **c** Proportion of cells in G2/M phase calculated from histogram **a** and **b**, respectively. * $p < 0.05$



experiments. Exposure of Ben-Men-1 cells to various concentrations of curcumin for 24 h significantly increased the expression of CDK1 and cyclin B1 as compared with DMSO-treated control cells. The critical regulatory step for activation of CDK1 appears to be dephosphorylation of CDK1 at Thr15 and Thr14. Western blot analysis using the phosphor-specific antibody against Thr15 revealed that phosphorylation level of CDK1 at Thr 15 increased after 24 h curcumin treatment. Failure to dephosphorylate CDK1 at Thr15 could be the cause of inactivation of CDK1/cyclin B1 complex, resulting in cell cycle arrest in G2/M. We next examined the effect of curcumin on other cell cycle regulatory proteins and observed that curcumin enhanced the expression of

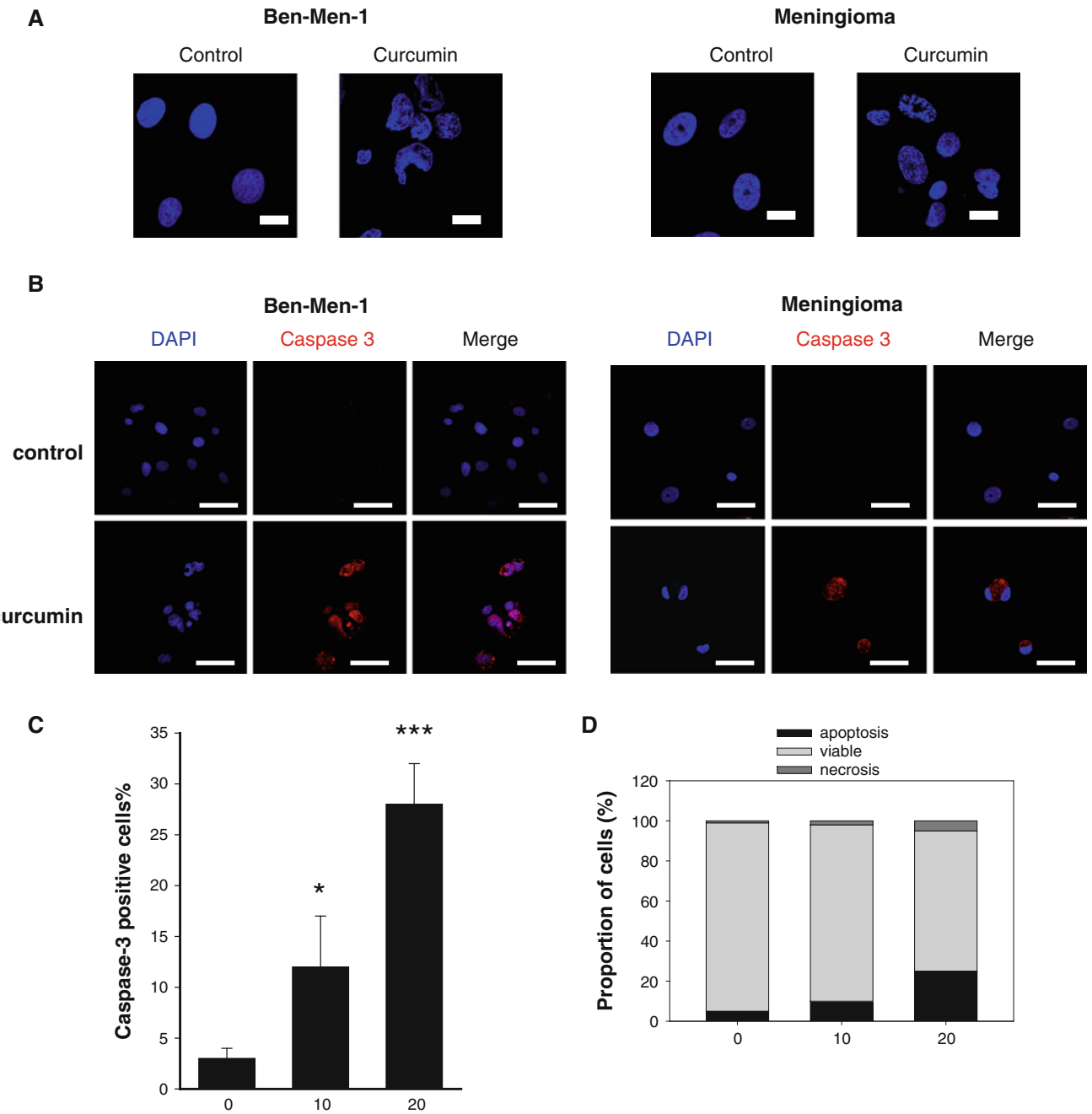


Fig. 4 Curcumin induces apoptosis in human meningioma cells. **a** Morphology of Ben-Men-1 cells (*left pictures*) and cells of a representative human meningioma (M10) in primary culture (*right pictures*) after treatment with vehicle (0.2 % DMSO in culture medium) or with curcumin (20 μM) for 24 h. Subsequent staining of cell nuclei with DAPI showed morphological changes of the nuclei in curcumin-treated cells indicating an onset of apoptosis. **b** Representative confocal microscopy images of Ben-Men-1 cells (*left pictures*) and cells of a human meningioma (M20, *right pictures*) which were treated with 20 μM curcumin and then stained for cleaved caspase-3 (*red*), a marker of apoptosis. *Blue* DAPI staining showing total nuclei. The *scale bars* in **a** and **b** are equal to 10 and 20 μM, respectively. **c** Quantitative data were obtained by counting the ratio of caspase-3 positive cells with DAPI stained cell population. * $p < 0.05$; *** $p < 0.001$. **d** Detection of viable (*light grey*), apoptotic (*black*) and necrotic (*dark grey*) Ben-Men-1 cells by FACS analysis after treatment with curcumin (10 and 30 μM) for 24 h. Curcumin dose dependently and significantly increased the proportion of cells undergoing apoptosis (cells staining immunopositive for Annexin V) without any significant induction of necrosis (cells staining immunopositive for 7-AAD)

cyclin-dependent kinase inhibitor p21. Protein expression of CDK2, cyclin D3, and cyclin E remained unaltered during the treatment (Fig. 3a).

To prove the relevance of the findings obtained from Ben-Men-1 cell lines in human meningiomas, the effect of curcumin on G2/M cell cycle related proteins were studied

by western blot in 6 primary meningioma cell cultures. The results obtained with the primary meningioma cell cultures were similar to the findings in Ben-Men-1 cells demonstrating that curcumin treatment led to the upregulation of cyclin B1, CDK1, p-CDK1-Thr15 and p21 in all 6 meningioma cell cultures studied (Fig. 3c shows a representative result).

To further investigate the expression level and cellular distribution of the cell cycle related proteins, an immunofluorescence experiment was carried out in curcumin treated Ben-Men-1 cells (Fig. 3b), as well as in 6 human primary meningioma cell cultures (representative confocal laser microscopy images from one meningioma culture are shown in Fig. 3d). In all cases the fluorescent signal of cyclin B1, CDK1, p-CDK1-Thr15 and p21 was increased after 24 h of treatment with 20 μM curcumin. CDK-1 staining was predominantly observed in the cytoplasm under basal conditions, but translocated to the nucleus in curcumin-treated cells. Nuclear staining of phosphorylated CDK1-Thr15 was also increased after curcumin treatment. These results confirm the findings obtained by western blotting and support the concept that the inactivation of cyclin B1/CDK1 complex is involved in curcumin-induced G2/M arrest in meningioma cells.

Curcumin induced apoptosis in human meningioma cells

The observed reduction in cell numbers after curcumin treatment (Fig. 1d) might not only be a consequence of suppression of proliferation, but also of induction of cell death. This speculation was supported by cell cycle analysis by FACS which showed that curcumin at higher dosages (20, 50 μM) increased the proportion of BEN-MEN-1 cells with signs of DNA degradation corresponding to cells undergoing apoptosis (Fig. 2a). In Ben-Men-1 cells, treatment with 20 μM curcumin for 24 h induced a proportion of 4.5 % apoptotic cells, which was increased to 18.3 % after application of 50 μM curcumin (Fig. 2a).

Since nuclear condensation and fragmentation are notable features of apoptosis, DAPI staining was performed in BEN-MEN-1 cells and 6 human meningioma cell cultures to study curcumin-induced changes in cell morphology. The pictures in Fig. 4a show apoptotic morphological changes of cell nuclei, reduction in cell number with apoptotic body formation, and cell shrinkage induced by treatment with 20 μM curcumin for 24 h in both Ben-Men-1 cells (left panel) and one representative example of a human meningioma in primary cell culture (right panel).

To further demonstrate the role of curcumin on meningioma cell apoptosis, two additional methods were applied in curcumin-treated meningioma cells. Apoptosis-associated DNA degradation was measured by staining active caspase-3 activities, and change of membrane-associated serine residues was determined by FACS analysis of Annexin V-FITC. In both Ben-Men-1 and primary human meningioma cells, immunoflorescence analysis showed a curcumin-induced positive staining in apoptosis-promoting cleaved caspase-3 (Fig. 4b). Quantitative data were obtained by counting the ratio of caspase-3 and DAPI stained cell population. The statistical data revealed a significant increase of caspase-3 positive cells after curcumin treatment compared with DMSO controls (Fig. 4c).

To discriminate between curcumin-induced apoptosis and necrosis, FACS analysis was performed with Ben-Men-1 cells treated with 20 μM curcumin for 24 h, and the proportions of Annexin V-immunopositive apoptotic and 7-AAD-immunopostive necrotic cells were measured. The results obtained for Ben-Men-1 cells showed that curcumin induced a significant increase in apoptotic cells accompanied by a corresponding decline in normal viable cells (Fig. 4d). No signs of necrosis in response to curcumin treatment were found indicating that curcumin has no non-specific cytotoxic effects on meningioma cells. Thus curcumin not only suppresses proliferation but also induces apoptosis in human meningioma cells.

Discussion

In the present paper we have shown for the first time that curcumin has potent anti-tumorigenic actions in meningioma cells in vitro by arresting cell growth and inducing apoptosis suggesting a putative relevance of this drug for the treatment of meningiomas.

Meningioma cells show numerous abnormalities that may play a role in the initiation and progression of this heterogeneous group of tumors. We and others could show that drugs targeting different abnormally expressed growth factors, neuropeptides, their receptors or the subsequent signaling cascades could reduce the proliferation in primary cell cultures of meningiomas in vitro [13, 19, 21]. However, when tested in vivo, these drugs had very limited or no influence on meningioma progression and therefore, pharmacological treatment concepts are still of no significant clinical relevance for the routine therapy of meningiomas [2, 13, 14].

Curcumin, a polyphenolic herbal compound and major component of the curry spice has potent anti-tumorigenic activities and is under investigation in clinical trials for its therapeutic potential for the treatment of different types of cancer [22, 23]. Curcumin is a classical multi-targeting drug as it affects multiple extra- and intracellular components in tumor cells among them proteins of the extracellular matrix, structural proteins, growth factors/receptors, signaling proteins and transcription factors [15, 16, 23–25]

many of which are altered in meningioma cells. Thus curcumin might represent an ideal candidate to concomitantly target multiple abnormally expressed factors in meningioma cells in order to suppress proliferation and to induce apoptosis.

The application of curcumin for 24–48 h strongly suppressed the proliferation of cells from 19 benign and 2 more aggressive WHO-grade II meningiomas. Whether or not malignant WHO-grade III meningiomas, for which alternate treatment options are urgently needed, will also respond to curcumin treatment has to be clarified in future studies. In several other types of benign and aggressive neoplasias, among them intracranial tumors, curcumin has also shown strong anti-proliferative effects but in most cases these findings have been obtained only with representative tumor cell lines of the corresponding neoplasias [23–25]. Here we could demonstrate that curcumin did not only suppress the proliferation of Ben-Men-1 meningioma cells but also of all primary human meningioma cell cultures investigated underlining the putative clinical relevance of our findings.

The growth of meningioma cells was arrested at the G2/M phase similar to other reports from most of the other tumor cell types in which the anti-proliferative action of curcumin had been studied [20, 26–32]. Curcumin has been shown to affect different G2/M transition regulating cyclins, cyclin dependent kinases (CDK) and CDK inhibitors [33]. In the present paper curcumin treatment enhanced cyclin B1, CDK1, pCDK1(Thr15) and the cyclin-dependent kinase inhibitor p21 protein levels which is consistent with G2/M blockade. However, curcumin was also recently reported to suppress onset of mitosis by binding to factors involved in the mitotic spindle formation such as Aurora A or Cdc27 [31, 34]. Whether this mode of action is also involved in the G2/M arrest by curcumin in meningioma cells needs to be clarified.

Many studies have shown that prolonged cell cycle arrest of tumor cells in G2/M will finally induce apoptosis [33]. Therefore, in most tumor cell types curcumin treatment led to apoptosis which is characterized by typical cellular changes among them the formation of the apoptosome complex, the collapse of the mitochondrial membrane potential, activation of caspase-3 and down-regulation of Bcl-2 [28, 32–36]. In Ben-Men-1 meningioma cells and all primary meningioma cell cultures studied, curcumin induced classical signs of apoptosis such as nuclear fragmentation, change of membrane-associated serine residues and cleaved caspase-3 up-regulation. Whether autophagy, an alternate form of curcumin-induced cell death characterized by an increase in light chain 3 (LC3) protein [37–40], is also induced by curcumin in meningioma cells remains to be studied. No significant necrosis was induced by curcumin indicating no unspecific cytotoxic effects of the drug.

Our in vitro findings suggest that curcumin could be a putative drug for the treatment of meningiomas. However, the efficacy of curcumin in vivo is limited by its poor bioavailability as it is rapidly degraded in the liver and excreted by the kidney and therefore has a biological half-life of only few hours [41]. Thus in clinical trials, doses of curcumin that were effective in meningioma cells in the present work, were not or at best transiently achieved at the tumor site even after the oral administration of high amounts (up to 12 g per day) of curcumin for the treatment of gastrointestinal tumors [41–44]. This suggests that the native drug is a poor candidate for the chemotherapy of tumors. To overcome this problem, an increasing number of curcumin derivatives, curcumin analogs and different curcumin formulations, among them injectable curcumin-releasing microparticles have recently been developed [30, 38, 45–48]. These curcuminoids combine high efficacy and enhanced biostability and may thus be better suitable for the treatment of tumors, among them meningiomas, in the near future. We are currently planning to test this in meningioma xenografts in nude mice in which the anti-tumorigenic effect of more stable curcuminoids will be studied in vivo.

In summary, we have shown that the multi-targeting drug curcumin has potent anti-proliferative and pro-apoptotic actions in meningioma cells. More stable curcuminoids, which are actually under development and clinical testing, might therefore represent putative candidates for the chemotherapy of meningiomas.

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Conflicts of interest The authors declare that they have no conflicts of interest.

References

1. Mawrin C, Perry A (2010) Pathological classification and molecular genetics of meningiomas. J Neurooncol 99:379–391
2. Choy W, Kim W, Nagasawa D, Stramotas S, Yew A, Gopen Q, Parsa AT, Yang I (2011) The molecular genetics and tumor pathogenesis of meningiomas and the future directions of meningioma treatments. Neurosurg Focus 30:E6
3. Marosi C, Hassler M, Roessler K, Reni M, Sant M, Mazza E, Vecht C (2008) Meningioma. Crit Rev Oncol Hematol 67:153–171
4. Louis DN, Scheithauer BW, Budka H, von Deimling A, Kepes JJ (2000) Meningiomas. In: Kleihues P, Cavenee WK (eds) World health organization classification of tumours. Pathology and genetics of tumours of the nervous system. IARC Press, Lyon, pp 176–184
5. Ruttledge MH, Sarrazin J, Rangaratnam S, Phelan CM, Twist E, Merel P, Delattre O, Thomas G, Nordenskjold M, Collins VP (1997) Evidence for the complete inactivation of the NF2 gene in the majority of sporadic meningiomas. Nat Genet 6:180–184

6. Gutmann DH, Donahoe J, Perry A, Lemk N, Gorse K, Kittiniyom K, Rempel SA, Gutierrez JA, Newsham IF (2000) Loss of DAL-1, a protein 4.1-related tumor suppressor, is an important early event in the pathogenesis of meningiomas. Hum Mol Genet 9:1495–1500
7. Smith MJ, O'Sullivan J, Bhaskar SS, Hadfield KD, Poke G, Caird J, Sharif S, Ecles D, Fitzpatrick D, Rawluk D, du Plessis D, Newman WG, Evans DG (2013) Loss-of-function mutations in SMARCE1 cause an inherited disorder of multiple spinal meningiomas. Nat Genet 45:295–298
8. Clark VE, Erson-Omay EZ, Serin A, Yin J, Cotney J, Ozduman K, Avsar T, Li J, Murray PB, Henegariu O, Yilmaz S, Günel JM et al (2013) Genomic analysis of non-NF2 meningiomas reveals mutations in TRAF7, KLF4, AKT1, and SMO. Science 339:1077–1080
9. Perry A, Cai DX, Scheithauer BW, Swanson PE, Lohse CM, Newsham IF, Weaver A, Gutmann DH (2000) Merlin, DAL-1, and progesterone receptor expression in clinicopathologic subsets of meningioma: a correlative immunohistochemical study of 175 cases. J Neuropathol Exp Neurol 59:872–879
10. Bostrom J, Meyer-Puttlitz B, Wolter M, Blaschke B, Weber RG, Lichter P, Ichimura K, Collins VP, Reifenberger G (2001) Alterations of the tumor suppressor genes CDKN2A ($p16^{INK4a}$), $p14^{ARF}$, CDKN2B ($p15^{INK4b}$), and CDKN2C ($p18^{INK4c}$) in atypical and anaplastic meningiomas. Am J Pathol 159:661–669
11. Dutour A, Kumar U, Panetta R, Quafik L, Fina F, Sasi R, Patel YC (1998) Expression of somatostatin receptor subtypes in human brain tumors. Int J Cancer 76:620–627
12. Barresi V (2011) Angiogenesis in meningiomas. Brain Tumor Pathol 28:99–106
13. Norden AD, Drappatz J, Wen PY (2007) Targeted drug therapy for meningiomas. Neurosurg Focus 23:E12
14. Wen PY, Quant E, Drappatz J, Beroukhim R, Norden AD (2010) Medical therapies for meningiomas. J Neurooncol 99:365–378
15. Kunnumakkara AB, Anand P, Aggarwal BB (2008) Curcumin inhibits proliferation, invasion, angiogenesis and metastasis of different cancers through interaction with multiple cell signaling proteins. Cancer Lett 269:199–225
16. Gupta SC, Kim JH, Prasad S, Aggarwal BB (2010) Regulation of survival, proliferation, invasion, angiogenesis and metastasis of tumor cells through modulation of inflammatory pathways by nutraceuticals. Cancer Metastasis Rev 29:405–434
17. James MF, Han S, Polizzano C, Plotkin SR, Manning BD, Stemmer-Rachaminmov AO, Gusella JF, Ramesh V (2009) NF2/merlin is a novel negative regulator of mTOR complex 1, and activation of mTORC1 is associated with meningioma and schwannoma growth. Mol Cell Biol 29:4250–4261
18. Angelo LS, Wu YJ, Meng F, Sun M, Kopetz S, McCutcheon IE, Slopis JM, Kurzrock R (2011) Combining curcumin (diferuloylmethane) and heat shock protein inhibition for neurofibromatosis 2 treatment: analysis of response and resistance pathways. Mol Cancer Ther 10:2094–2103
19. Pagotto U, Arzberger T, Hopfner U, Sauer J, Renner U, Newton CJ, Lange M, Uhl E, Weindl A, Stalla GK (1995) Expression and localization of endothelin-1 and endothelin receptors in human meningiomas: evidence for a role in tumoral growth. J Clin Invest 96:2017–2025
20. Schaaf C, Shan B, Buchfelder M, Losa M, Kreutzer J, Rachinger W, Stalla GK, Schilling T, Arzt E, Perone MJ, Renner U (2009) Curcumin acts as anti-tumorigenic and hormone-suppressive agent in murine and human pituitary tumour cells in vitro and in vivo. Endocr Rel Cancer 16:1339–1350
21. Tichomirowa M, Theodoropoulou M, Daly AF, Yassourides A, Hansen S, Lu J, Lange M, Goldbrunner RH, Stalla GK, Renner U (2008) Toll-like receptor 4 is expressed in meningiomas and mediates the antiproliferative action of paclitaxel. Int J Cancer 123:1956–1963
22. Shezad A, Wahid F, Lee YS (2010) Curcumin in cancer chemoprevention: molecular targets, pharmacokinetics, bioavailability, and clinical trials. Arch Pharm Chem Life Sci 9:489–499
23. Gupta SC, Prasad S, Kim JH, Patchva S, Webb LJ, Priyadarsini IK, Aggarwal BB (2011) Multitargeting by curcumin as revealed by molecular interaction studies. Nat Prod Rep 28:1937–1955
24. Yadav VR, Aggarwal BB (2011) Curcumin: a component of the golden spice, targets multiple angiogenic pathways. Cancer Biol Ther 11:236–241
25. Sung B, Prasad S, Yadav VR, Aggarwal BB (2012) Cancer cell signaling pathways targeted by spice-derived nutraceuticals. Nutr Cancer 64:173–197
26. Schaaf C, Shan B, Onofri C, Stalla GK, Arzt E, Schilling T, Perone MJ, Renner U (2010) Curcumin inhibits the growth, induces apoptosis and modulates the function of pituitary folliculostellate cells. Neuroendocrinology 91:200–210
27. Aoki H, Takada Y, Kondo S, Sawaya R, Aggarwal BB, Kondo Y (2007) Evidence that curcumin suppresses the growth of malignant gliomas in vitro and in vivo through induction of autophagy: role of Akt and extracellular signal-regulated kinase signaling pathways. Mol Pharmacol 72:29–39
28. Weir NM, Selvendiran K, Kutala VK, Tong L, Vishwanath S, Rajaram M, Tridandapani S, Anant S, Kuppusamy P (2007) Curcumin induces G2/M arrest and apoptosis in cisplatin-resistant human ovarian cancer cells by modulating Akt and p38 MAPK. Cancer Biol Ther 6:178–184
29. Lee SJ, Krauthauser C, Maduskuie V, Fawcett PT, Olson JM, Rajasekaran SA (2011) Curcumin-induced HDAC inhibition and attenuation of medulloblastoma growth in vitro and in vivo. BMC Cancer 11:144–152
30. Lim KJ, Bisht S, Bar EE, Maitra A, Eberhart CG (2001) A polymeric nanoparticle formulation of curcumin inhibits growth, clonogenicity and stem-like fraction in malignant brain tumors. Cancer Biol Ther 11:464–473
31. Liu H-S, Ke C-S, Cheng H-C, Huang C-YF, Su C-L (2011) Curcumin-induced mitotic spindle defect and cell cycle arrest in human bladder cancer cells occurs partly through inhibition of Aurora A. Mol Pharmacol 80:638–648
32. Zanotto-Filho A, Braganhol E, Edelweiss M, Behr GA, Zanin R, Schröder R, Simoes-Pires A, Battastini AM, Moreira JC (2012) The curry spice curcumin selectively inhibits cancer cell growth in vitro and in a preclinical model of glioblastoma. J Nutr Biochem 23:591–601
33. Karunagaran D, Joseph J, Kumar TR (2007) Cell growth regulation. Adv Exp Med Biol 595:245–268
34. Lee SJ, Langhans SA (2012) Anaphase-promoting complex/cyclosome protein Cdc27 is a target for curcumin-induced cell cycle arrest and apoptosis. BMC Cancer 12:44–56
35. Shishodia S, Amin HM, Lai R, Aggarwal BB (2005) Curcumin (diferuloylmethane) inhibits constitutive NF-kappaB activation, induces G1/S arrest, suppresses proliferation, and induces apoptosis in mantle cell lymphoma. Biochem Pharmacol 70:700–713
36. Srivastava RK, Chen Q, Siddiqui I, Sarva K, Shankar S (2007) Linkage of curcumin-induced cell cycle arrest and apoptosis by cyclin-dependent kinase inhibitor p21/waf1/cip. Cell Cycle 6:2953–2961
37. Teiten M-H, Gaascht F, Cronauer M, Henry E, Dicato M, Diederich M (2011) Anti-proliferative potential of curcumin in androgen-dependent prostate cancer cells occurs through modulation of the Wingless signaling pathway. Int J Cancer 38: 603–611
38. Wu JC, Lai CS, Badmaev V, Nagabhushanam K, Ho CT, Pan MH (2011) Tetrahydrocurcumin, a major metabolite of curcumin, induced autophagic cell death through coordinative modulation of PI3 K/Akt-mTOR and MAPK signaling pathways in human leukemia HL 60 cells. Mol Nutr Food Res 55:1646–1654

39. Jia YL, Li J, Qin ZH, Liang ZQ (2009) Autophagic and apoptotic mechanisms of curcumin-induced death in K562 cells. J Asian Nat Prod Res 11:918–928
40. Qian H, Yang Y, Wang X (2011) Curcumin enhanced adriamycin-indued human liver-derived Hepatoma G2 cell death through activation of mitochondria-mediated apoptosis and autophagy. Eur J Pharmacol Sci 43:125–131
41. Anand P, Kunnumakkara AB, Newman RA, Aggarwal BB (2007) Bioavailability of curcumin: problems and promises. Mol Pharm 4:807–818
42. Cheng AL, Hsu CH, Lin JK, Hsu MM, Yo YF, Shen TS, Ko JY, Lin JT, Lin BR, Ming-Shiang W, Yu HS, Jee SH et al (2001) Phase I clinical trial of curcumin, a chemopreventive agent in patients with high-risk of pre-malignant lesions. Anticancer Res 21:2895–2900
43. Sharma RA, Euden SA, Patton SL, Cooke DN, Shafayat A, Hewitt HR, Marczylo TH, Morgan B, Hemingway D, Plummer SM, Pirmohamed M, Gescher AJ et al (2004) Phase I clinical trial of curcumin: biomarkers of systemic activity and compliance. Clin Cancer Res 10:6847–6854
44. Garcea G, Jones DJ, Singh R, Dennison AR, Farmer PB, Sharma RA, Steward WP, Gescher AJ, Berry DP (2004) Detection of curcumin and its metabolites in hepatic tissue and portal blood of patients following oral administration. Br J Cancer 90:1011–1015
45. Thomas SL, Zhong D, Zhou W, Malik S, Liotta D, Snyder JP, Hamel E, Giannakakou P (2008) EF24, a novel curcumin analog, disrupts the microtubule cytoskeleton and inhibits HIF-1. Cell Cycle 7:2409–2417
46. Li J, Wang Y, Yang C, Wang P, Oelschlager DK, Zheng Y, Tian D-A, Grizzle WE, Buchsbaum DJ, Wan M (2009) Polyethylene glycosylated curcumin conjugate inhibits pancreatic cancer cell growth through inactivation of Jab1. Mol Pharmacol 76:81–90
47. Selvendiran K, Kuppusamy ML, Bratasz A, Tong L, Rivera BK, Rink C, Sen CK, Kalai T, Hideg K, Kuppusamy P (2009) Inhibition of vascular smooth-muscle cell proliferation and arterial restenosis by HO-3867, a novel synthetic curcumoid, through up-regulation of PTEN expression. J Pharmacol Exp Ther 329:959–966
48. Shahani K, Swaminathan SK, Freeman D, Blum A, Ma L, Panyam J (2010) Injectable sustained release microparticles of curcumin: a new concept for cancer chemoprevention. Cancer Res 70:4443–4452