Curcumin acts as anti-tumorigenic and hormone-suppressive agent in murine and human pituitary tumour cells *in vitro* and *in vivo*

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Abstract

Curcumin (diferuloylmethane) is the active ingredient of the spice plant *Curcuma longa* and has been shown to act anti-tumorigenic in different types of tumours. Therefore, we have studied its effect in pituitary tumour cell lines and adenomas. Proliferation of lactosomatotroph GH3 and somatotroph MtT/S rat pituitary cells as well as of corticotroph AtT20 mouse pituitary cells was inhibited by curcumin in monolayer cell culture and in colony formation assay in soft agar. Fluorescence-activated cell sorting (FACS) analysis demonstrated curcumin-induced cell cycle arrest at G2/M. Analysis of cell cycle proteins by immunoblotting showed reduction in cyclin D_1, cyclin-dependent kinase 4 and no change in p27^{kip}. FACS analysis with Annexin V-FITC/7-aminoactinomycin D staining demonstrated curcumin-induced early apoptosis after 3, 6, 12 and 24 h treatment and nearly no necrosis. Induction of DNA fragmentation, reduction of Bcl-2 and enhancement of cleaved caspase-3 further confirmed induction of apoptosis by curcumin. Growth of GH3 tumours in athymic nude mice was suppressed by curcumin *in vivo*. In endocrine pituitary tumour cell lines, GH, ACTH and prolactin production were inhibited by curcumin. Studies in 25 human pituitary adenoma cell cultures have confirmed the anti-tumorigenic and hormone-suppressive effects of curcumin. Altogether, the results described in this report suggest this natural compound as a good candidate for therapeutic use on pituitary tumours.

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Introduction

Clinically, active tumours of the anterior pituitary represent the second most frequent form of intracranial neoplasms with a prevalence of about 300 per one million inhabitants per year (Davis *et al*. 2001). The monoclonal pituitary tumours are mostly benign, endocrine-active or -inactive adenomas; pituitary carcinomas are extremely rare. The clinical symptoms

of the tumours are caused by their space-occupying lesion (e.g. headache, visual field loss) and, in case of endocrine-active adenomas, by the excessive hormone secretion of the tumour cells (Ezzat & Asa 2006). GH-secreting tumours cause gigantism in children and acromegaly in adults (Chanson & Salenave 2008). ACTH-producing adenomas induce Cushing's disease and prolactin (PRL)-releasing tumours cause the

symptoms of hyperprolactaemia (Newell-Price *et al.* 2006, Mancini *et al.* 2008). Thyreotrophin- and gonadotrophin-producing adenomas are rare (Ezzat & Asa 2006). A life-long pharmacological treatment with dopamine agonists (e.g. bromocriptine, cabergoline) is highly effective in prolactinomas (Mancini *et al.* 2008). In patients with acromegaly, treatment with somato-statin analogues (e.g. octreotide, lanreotide and pasireotide) or with the GH receptor antagonist (e.g. pegvisomant) normalizes the GH- and insulin-like growth factor 1 levels in about 50% of the affected patients (Chanson & Salenave 2008). Other pituitary adenoma types and tumours from pharmacotreatment-resistant patients have to be removed by surgery. Since the latter is often not associated with complete cure of the patients, additional pharmacological concepts for the treatment of pituitary adenomas would be desirable.

It is assumed that plants contain a big reservoir of drugs with anti-tumorigenic properties (Corson & Crews 2007), but so far only few plant-derived substances such as vincristine or paclitaxel have achieved clinical relevance as chemotherapeutic agents (Gidding *et al.* 1999, Fitzpatrick & Wheeler 2003). However, during the recent years, a growing number of plant-derived drugs with anti-tumorigenic activities have been detected, one among them is curcumin (diferuloylmethane), a biphenolic natural product and the active component of turmeric (*Curcuma longa*), which gives Indian curry its yellow colour and distinctive taste (Aggarwal *et al.* 2007). Having been used as a remedy in traditional Indian medicine for centuries, curcumin has recently been identified as an anti-cancer drug in various types of cancer (Shishodia *et al.* 2007, Hatcher *et al.* 2008) but has also been demonstrated to exhibit anti-depressant, anti-oxidative, anti-inflammatory and neuroprotective actions (Singh 2007). The anti-tumorigenic properties of curcumin involve both anti-proliferative and pro-apoptotic effects on tumour cells (Sa & Das 2008, Shankar *et al.* 2008). Regarding its mechanism of action, curcumin was shown to act at multiple levels. Curcumin modified histone acetylation, which in turn affected gene expression (Kunnumakkara *et al.* 2008), affected cyclin and cyclin-dependent kinases (CDKs), modulated phosphatidylinositol 3 kinase (PI3K)/Akt and MAP kinase signal pathways, affected apoptotic proteins (Bcl-2 and Bax), reduced secretion of angiogenic factors and modified the expression of transcription factors (e.g. down-regulated NF-κB; Sandur *et al.* 2007, Barnes 2008, Carroll *et al.* 2008, Chadalapaka *et al.* 2008, Sa & Das 2008).

Curcumin is currently tested in several phase II clinical studies for its efficacy in the treatment of various diseases such as colon and pancreatic cancers (Corson & Crews 2007, Johnson & Mukhtar 2007, Dhillon *et al.* 2008).

In a recent paper, it has been shown that curcumin inhibited proliferation, induced apoptosis and sup-pressed hormone secretion in the lactosomatotroph GH3 rat pituitary tumour cell line (Miller *et al.* 2008). The aim of the present work is to study anti-tumorigenic actions of curcumin in other rodent endocrine pituitary tumour cells. Moreover, we explored for the first time about the role of curcumin *in vivo* in experimentally induced pituitary tumours of nude mice and in primary cell cultures of human pituitary adenomas.

Materials and methods

Materials

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

Cell culture and curcumin stimulation

Lactosomatotroph GH3 and somatotroph MtT/S rat pituitary cells as well as corticotroph AtT20 mouse pituitary cells were cultured at 5% CO_2 and 37 °C in DMEM (pH 7.3) supplemented with 10% FCS, 2.2 g/l $NaHCO_3$, 10 mM HEPES and 2 mM glutamine, 2.5 mg/l amphotericin B, 10^5 U/l penicillin–strepto-mycin, 5 mg/l insulin, 5 mg/l transferrin, 20 µg/l sodium selenite and 30 pM tri-iodothyronine (Henning, Berlin, Germany).

For primary human pituitary tumour cell culture, adenoma tissue was obtained by transsphenoidal surgery from 25 patients with various types of pituitary adenomas as indicated in Table 1. Dis-persion of the tumour tissues and subsequent cultivation of the tumour cells were performed in the same way as for pituitary cell lines and as previously described (Renner *et al.* 1998). Due to the limited amount of human pituitary tumour tissue available, not all experiments could be done in parallel in primary human pituitary tumour cell cultures (see Table 1).

For *in vitro* experiments, a stock solution of 10 mM curcumin (prepared in DMSO) was diluted with cell

Table 1 Overview about human pituitary adenomas studied and results obtained after treatment with curcumin in primary cell culture

Tumour[a]	Hormones	Sex/age	Proliferation[b]	Apoptosis[c]	Hormone secretion	
					% Inhibition[d]	Basal[e]
LST1	PRL	M/66	$59.8 \pm 5.3^{\dagger}$	ND	$65.1 \pm 6.0^{*}$	855.7 ± 19.3
	GH	M/66	$59.8 \pm 5.3^{\dagger}$	ND	$77.5 \pm 3.0^{*}$	1226.3 ± 30.0
LT1	PRL	F/60	ND	ND	99.8 ± 1.4	62.6 ± 0.3
ST1	GH	F/41	$69.1 \pm 1.5^{*}$	ND	ND	ND
ST2	GH	F/57	ND	ND	$77.0 \pm 4.5^{*}$	1496.5 ± 47.5
ST3	GH	F/45	ND	ND	$60.4 \pm 8.1^{*}$	106.1 ± 7.6
ST4	GH	M/35	ND	ND	$56.5 \pm 5.2^{*}$	230.7 ± 20.7
ST5	GH	M/32	ND	ND	94.0 ± 4.9	301.7 ± 37.0
ST6	GH	F/53	99.0 ± 3.5	ND	$88.0 \pm 0.3^{*}$	87.9 ± 5.3
ST7	GH	F/42	$61.2 \pm 1.9^{\dagger}$	$3.19 \pm 0.09^{*}$	$55.2 \pm 2.7^{\dagger}$	436.5 ± 8.2
ST8	GH	M/56	ND	$2.37 \pm 0.41^{*}$	$80.1 \pm 3.1^{*}$	1056.5 ± 73.2
ST9	GH	M/38	$16.7 \pm 5.8^{\dagger}$	ND	$62.9 \pm 9.5^{*}$	1139.1 ± 113.0
TT1	TSH	F/64	ND	ND	$60.0 \pm 2.9^{\dagger}$	9.80 ± 0.32
TT2	TSH	M/28	93.3 ± 3.8	ND	ND	ND
NT1	None	M/70	$29.7 \pm 1.6^{\dagger}$	ND	–	–
NT2	None	F/72	$65.9 \pm 12.5^{*}$	ND	–	–
NT3	None	M/68	$49.1 \pm 6.5^{\dagger}$	$10.52 \pm 1.12^{*}$	–	–
NT4	None	M/66	$78.7 \pm 4.0^{*}$	ND	–	–
NT5	None	M/70	$78.6 \pm 11.5^{*}$	ND	–	–
NT6	None	M/64	86.3 ± 15.2	ND	–	–
NT7	None	F/71	$52.6 \pm 4.1^{\dagger}$	$3.70 \pm 0.27^{*}$	–	–
NT8	None	F/72	ND	$4.45 \pm 0.47^{*}$	–	–
NT9	None	M/55	$48.1 \pm 6.0^{\dagger}$	$2.06 \pm 0.01^{*}$	–	–
NT10	None	M/41	$11.7 \pm 1.8^{\dagger}$	ND	–	–
NT11	None	M/51	$52.2 \pm 6.4^{\dagger}$	ND	–	–
NT12	None	M/70	$30.9 \pm 7.1^{\dagger}$	ND	–	–

F, female; M, male; ND, not determined. $^{*}P < 0.05$; $^{\dagger}P < 0.01$ versus corresponding untreated cells.
[a]LST, lactosomatotroph tumour; LT, lactotroph tumour; ST, somatotroph tumour; TT, thyreotroph tumour; NT, non-functioning tumour.
[b]Maximum inhibition of [3H]-thymidine incorporation expressed as percentage of untreated cells ($= 100\%$).
[c]Apoptosis was determined by cell death ELISA and is expressed as maximum fold stimulation of apoptosis in untreated cells.
[d]Maximum inhibition of hormone secretion expressed as percentage of untreated cells ($= 100\%$).
[e]Hormone concentration (ng/ml) in cell culture supernatants of untreated human pituitary adenoma cells after 24 h.

culture medium to obtain concentrations of 0.5–30 μM curcumin for cell treatments. During curcumin application for various time periods as indicated, light exposure was reduced as much as possible. Controls with 0.3% DMSO corresponding to the amount present in the highest curcumin dosage of 30 μM were performed in each experiment to exclude effects of this solvent.

In vitro proliferation assays

To study the effects of curcumin on the proliferation of pituitary tumour cell lines and human pituitary adenoma cells, [³H]thymidine incorporation experiments were performed as previously described (Renner *et al.* 1995, 1998). For all murine pituitary tumour cell lines, the effect of curcumin on cell growth was confirmed by direct cell counting using a cell size-adapted coulter counter.

Colony formation assay

The soft agar colony formation assay was used to assess the growth inhibitory effect of curcumin on GH3 cells in a three-dimensional *in vitro* model of cell culture. Non-pre-treated and curcumin-pre-treated (24 h, 30 μM) GH3 cells were seeded in soft agar. To this end, cells were suspended in 0.3% agar (noble agar, Sigma) solution consisting of 4.5 ml 2× DMEM medium (Invitrogen), 4.5 ml 1× DMEM medium and 2.25 ml FCS. The cells were plated in triplicate in 6-well plates (Nunc) coated with 0.5% agar at the density of 8000 cells per well and maintained at 37 °C for 14–21 days. Curcumin solution (30 μM, 1 ml per well) was added to non-pre-treated and pre-treated cells on top of the agar on the first day after seeding and was then renewed every 4 days. At the end of the treatment period, colonies were stained with 0.05% thiazolyl

blue tetrazolium bromide (Sigma) solution in 0.01 M PBS and photographed. Colony counting was performed with a special analysing tool from SC.O Lifesciences GmbH.

Experiments with athymic nude mice

Nude mice were housed in a pathogen-free environment at the Animal House Facility of The School of Natural Sciences (FCEyN), University of Buenos Aires. Experimental tumours were induced by injecting one million GH3 cells suspended in 20 µl PBS into a subdermal pouch in the flanks of adult male nude mice as previously described (Paez Pereda *et al.* 2003, Perez Castro *et al.* 2003). Curcumin treatment was started when the tumour volume had reached about 100 mm^3 in size. Curcumin was dissolved in 0.5 M NaOH–PBS and a total amount of 1 mg curcumin per mouse was administered i.p., 200 µl injection thrice a week. Vehicle-treated animals served as controls. The tumour volume was regularly determined as previously described (Paez Pereda *et al.* 2003, Perez Castro *et al.* 2003) until the animals were killed. The Ethical Committee on Animal Care and Use, University of Buenos Aires, Argentina had approved the animal studies.

Fluorescence-activated cell sorting analysis

Fluorescence-activated cell sorting (FACS) analysis was performed to determine the effect of curcumin on cell cycle arrest of pituitary tumour cell lines. 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 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 MtT/S and GH3 cells after treatment with 5–30 µM curcumin using Annexin V-FITC (Beckman Coulter) with commercially available kit (Beckman Coulter, IM3614). Apoptotic cells were defined as cells that stained positive for Annexin V-FITC binding on outer cell membranes, whereas 7-aminoactinomycin D (7-AAD)-positive cells were considered to be necrotic (Quinn *et al.* 2007). 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.

Detection of apoptosis by DNA fragmentation

In addition to FACS analysis, DNA fragmentation as an indicator of apoptosis was measured by a cell death detection ELISA (DNA Fragmentation Kit, Roche). After seeding and 24 h treatment with 5–30 µM curcumin, DNA was extracted from the cells, and DNA fragmentation activity was quantified according to the manufacturer's instructions.

Western blot analysis

To study the effects of curcumin on various cell cycle regulating and apoptosis-related proteins, GH3, AtT20 and MtT/S cells were stimulated with curcumin as indicated, and after protein extraction, western immunoblotting was performed as previously described (Paez-Pereda *et al.* 2003). Primary antibodies for the detection of Bcl-2, caspase-3, cyclin D1, p27^{kip1} (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 Ab (Millipore Corp., Billerica, MA, USA) was made in mouse. HRP-conjugated secondary antibodies against rabbit and mouse were all obtained from Santa Cruz Biotechnologies.

Hormone stimulation and measurement

To evaluate the effect of curcumin on hormone secretion, pituitary tumour cell lines as well as human endocrine-active adenoma cell cultures were stimulated with curcumin as indicated. ACTH, rat GH and rat PRL levels in the cell culture supernatants were analysed by RIA as described elsewhere (Stalla *et al.* 1989) with materials kindly provided by Dr A F Parlow from the National Hormone & Peptide Program (Torrance, CA, USA). Human GH was measured with Immulite 2500 chemiluminescence assay (Siemens, Munich, Germany), PRL and TSH with electrochemiluminescence immunoassay (Roche).

Statistical analysis

Each of the experiments was repeated at least thrice. The individual experiments were performed with quadruplicate wells. Results were analysed by one-factorial univariate ANOVA and followed by 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 $\pm$ s.e.m.

Results

Effect of curcumin on pituitary tumour cell proliferation *in vitro* and *in vivo*

In two *in vitro* proliferation assays, [³H] thymidine incorporation and determination of cell number, curcumin dose and time dependently inhibited growth of MtT/S, AtT20 and GH3 cells (Fig. 1A–D). MtT/S and AtT20 cells were most sensitive to curcumin treatment and significant growth suppression was already achieved at 5 μM curcumin. The inhibitory effect of curcumin was dose-dependent and concentrations of 20 and 30 μM significantly inhibited proliferation of all cell lines studied (Fig. 1A–D).

To test whether the growth inhibitory effect of curcumin is not only restricted to monolayer cell cultures, GH3 cells were used to assay the antiproliferative effect of curcumin in a three-dimensional soft agar cell culture model, which much closer than monolayer cell cultures mimic the *in vivo* situation. In one experiment, the cells were pre-treated with curcumin for 24 h to ensure maximal penetration of the cells with curcumin before seeding in soft agar, whereas, in the other experiment, GH3 cells were only treated after seeding, and thus curcumin could reach the cells in soft agar initially only by diffusion. In both the experiments, treatment with curcumin was then continued for 14 days. Curcumin dose dependently reduced the number of colonies formed in soft agar, and no difference in the degree of colony growth suppression was observed between non-pre-treated (Fig. 2A) and pre-treated (not shown) GH3 cells.

To study the inhibitory potential of curcumin on pituitary tumour progression *in vivo*, athymic nude mice bearing experimentally induced tumours were
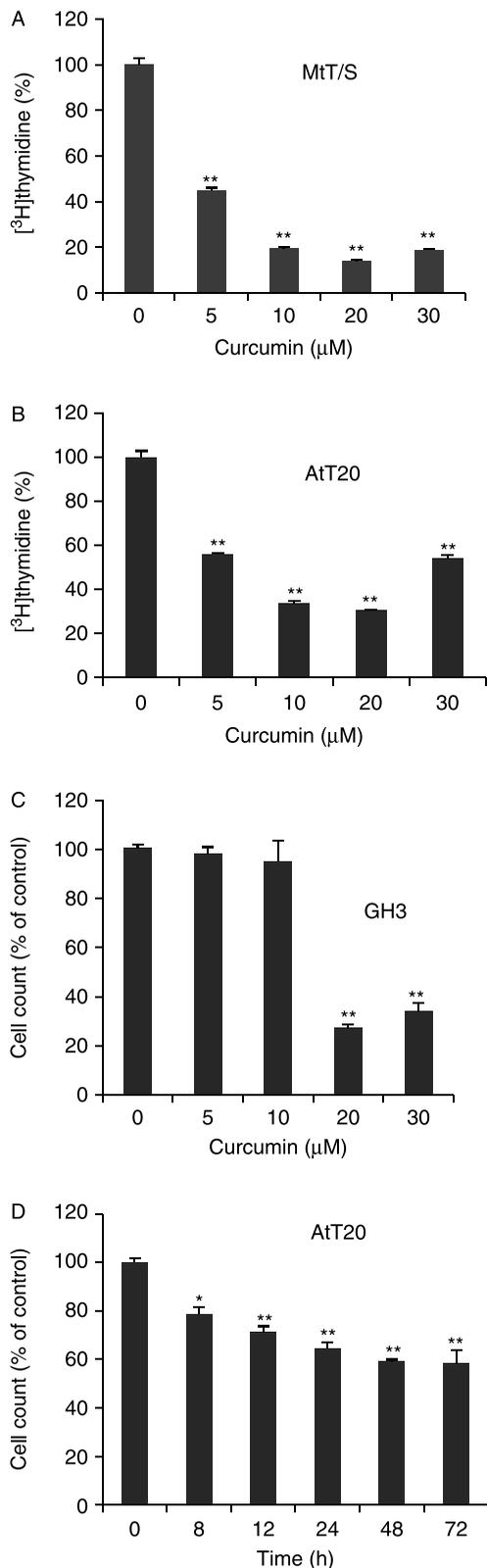
Figure 1 Effect of curcumin on growth of pituitary tumour cell lines. AtT20, MtT/S and GH3 cells were treated with 5–30 μM curcumin for up to 72 h, and effects on [³H]thymidine incorporation and cell numbers were determined. Representative figures show curcumin-induced inhibition of [³H]thymidine incorporation in MtT/S cells ((A) 24 h treatment) and AtT20 cells ((B) 48 h treatment) as well as reduction of cell numbers in GH3 cells ((C) 48 h treatment). For comparative reasons, in figures (A), (B) and (C), values are expressed as percentage of non-treated cells. In (D), an example of a time-course study is shown for AtT20 cells in which the gradual decline of proliferation over time after treatment with 10 μM curcumin is demonstrated. For each time point, the relative decline of cell numbers of curcumin-treated versus non-treated AtT20 cells is shown. *$P < 0.05$; **$P < 0.01$ versus non-treated cells.

used. To this end, GH3 cells were inoculated in the flanks of nude mice and curcumin treatment was started at a tumour size of ~ 100 mm^3. In comparison to vehicle-treated animals, injection of 1 mg curcumin per mouse/ thrice weekly induced a significant suppression of tumour size after 13 days. The difference in tumour size between vehicle- and curcumin-treated animals gradually increased until the end of the treatment period (Fig. 2B and C). Importantly, in this experiment, curcumin treatment started when the tumour volume had reached about 100 mm^3 in size resembling, in some way, the situation

of human pituitary tumours, where clinical symptoms appeared after the tumour had reached a considerable size. These results suggest that curcumin might have therapeutic effects on pituitary tumours.

Cell cycle arrest induced by curcumin treatment on pituitary tumour cell lines

Having demonstrated the anti-proliferative effect of curcumin in pituitary tumour cells, FACS analysis was performed to determine at which phase of the cell cycle curcumin induced growth arrest on GH3 and MtT/S cells. In parallel, the expression of key cell cycle regulating proteins was measured by western immunoblotting in extracts of curcumin-treated cells. Results of FACS analysis and western immunoblotting are shown for GH3 cells (Fig. 3), but similar results have been obtained for MtT/S pituitary tumour cells (not shown). Twenty-four-hour curcumin treatment induced a dose-dependent reduction in GH3 cells at G1 accompanied by an increase in cells at the G2/M phase (Fig. 3A), suggesting cell cycle arrest in G2/M phase. In extracts of GH3 cells, 24 h treatment with curcumin led to a down-regulation of cyclin D1 and CDK4 (Fig. 3B), which are both needed for promotion of the cells through the initial phase of G1. In contrast, expression of p27^{kip} was not affected by curcumin (Fig. 3C).

Curcumin induced apoptosis on pituitary tumour cell lines

The observed reduction in cell numbers and tumour size after curcumin treatment (Figs 1 and 2) might not only be a consequence of suppression of proliferation, but also induction of cell death. Thus, two different methods were applied to detect apoptosis
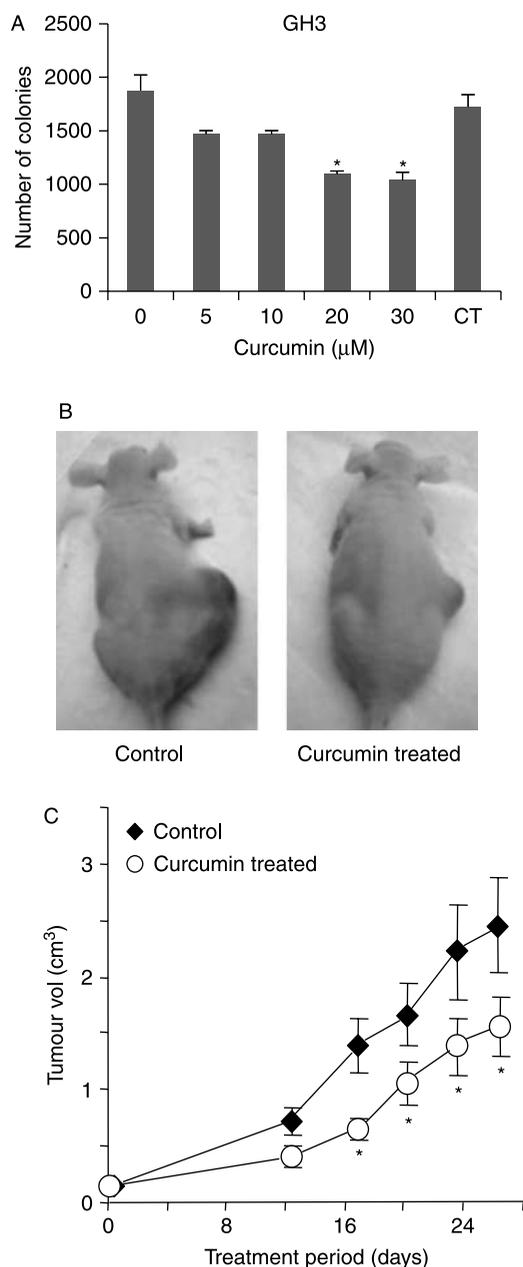
Figure 2 Effect of curcumin on GH3 cell colony formation in soft agar (A) and on GH3 cell tumour growth in nude mice (B and C). (A) GH3 cells were seeded in soft agar in 6-well culture plates and treated with 5–30 μM curcumin or were not treated (0). Curcumin solutions (1 ml) were added on the first day after seeding and were then renewed every 4 days. After 14 days, colony numbers were determined. Since curcumin solutions contained small amounts of DMSO, control cells (CT) were treated with the highest concentration of DMSO applied. Whereas the latter had no effect on colony formation, curcumin dose dependently suppressed the number of GH3 cell colonies. (B and C) Athymic mice bearing GH3 tumour cells were treated with curcumin for 4 weeks (1 mg per animal/ thrice weekly) or vehicle (control; six animals per group) after the tumour size has reached 100 mm^3. Suppression of tumour growth by curcumin was evident after 13 days, and the difference in tumour size between curcumin- and vehicle-treated mice gradually increased over treatment time. Representative tumours on day 27 are shown in (B) and, in (C), the development of mean tumour size during the treatment period is documented. *$P<0.05$; **$P<0.01$ versus non-treated cells (A) or vehicle controls (B and C) respectively.
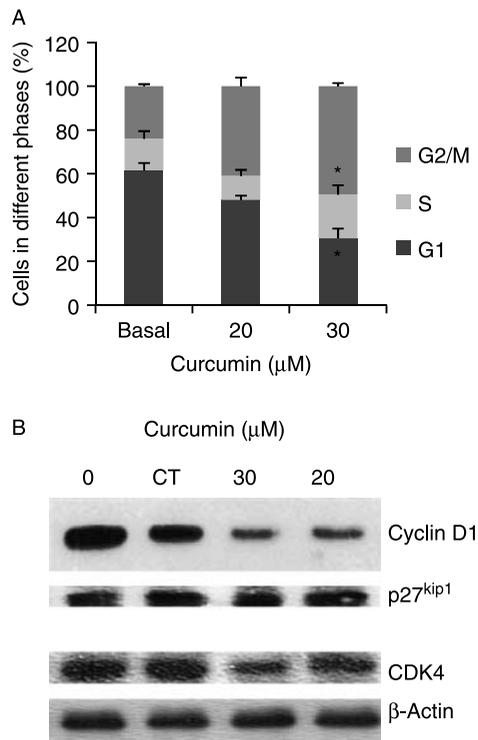
Figure 3 Effect of curcumin on the cell cycle and cell cycle regulating proteins in GH3 cells. After stimulation of GH3 cells with the indicated curcumin concentrations for 24 h, the proportion of cells at different cell cycle phases was determined by FACS analysis (A), and alterations in the expression of cell cycle regulating proteins were measured by western immunoblotting (B). (A) Curcumin dose dependently and significantly enhanced the proportion of GH3 cells in G2/M phase and reduced cell numbers in G1 phase indicating a curcumin-induced growth arrest in G2/M phase. (B) Studies on the effect of curcumin on key cell cycle regulating proteins showed a dose-dependent suppression of cyclin D1 and CDK4 but no effect on p27^{kip1} in GH3 cells. CT, control with DMSO. *$P < 0.05$ versus non-treated (basal) cells.

in curcumin-treated GH3, MtT/S and AtT20 cells. Apoptosis-associated DNA degradation was measured by ELISA, and change of membrane-associated serine residues was determined by FACS analysis of Annexin V-FITC.

Curcumin (5–30 μM) dose dependently increased apoptotic DNA fragmentation in all cell lines as measured by ELISA. In MtT/S cells, 30 μM curcumin induced a maximum 9.57 ± 0.71 fold stimulation of apoptosis versus non-treated cells, whereas a 3.01 ± 0.13 and 4.09 ± 0.12 fold stimulation of apoptosis was measured in GH3 and AtT20 cells respectively (in Fig. 4A, results are shown for MtT/S cells).

In AtT20, GH3 and MtT/S cells, western immunoblotting analysis showed a curcumin-induced, dose-dependent reduction in anti-apoptotic-acting Bcl-2 and an increase in apoptosis-promoting cleaved

caspase-3. In Fig. 4B, immunoblots are shown only for MtT/S cells, but similar results were obtained for AtT20 and GH3 cells (not shown).

To discriminate between curcumin-induced apoptosis and necrosis, FACS analysis was performed with MtT/S and GH3 cells treated with 30 μM curcumin for 6, 12 and 24 h, and the proportions of Annexin V-immunopositive apoptotic and 7-AAD-immunopostive necrotic cells were measured. The results obtained for MtT/S cells (Fig. 4C) were similar to those for GH3 cells (not shown). Curcumin induced a time-dependent, significant increase in apoptotic cells accompanied by a corresponding decline in normal viable cells (Fig. 4C). No signs of necrosis in response to curcumin treatment were found indicating that curcumin has no non-specific cytotoxic effects on pituitary tumour cells. Thus, curcumin is not only suppressing proliferation but is also inducing apoptosis in pituitary tumour cells.

Suppression of hormone secretion by curcumin

Small and slowly growing, endocrine-active adenomas, e.g. corticotroph microadenomas or microprolactinomas, represent a considerable proportion of all pituitary tumours. In patients with such tumours, normalization of the excessive and pathophysiological hormone secretion is the primary therapeutic goal. When testing the hormone-suppressive activity of curcumin, we could demonstrate that curcumin significantly reduced ACTH secretion by corticotroph AtT20 cells (Fig. 5A). We could also confirm the recently reported (Miller *et al*. 2008) inhibitory effect of curcumin on PRL and GH secretion in GH3 cells (Fig. 5B and C). Thus, curcumin has the potential to reduce the excessive hormone production of endocrine-active pituitary tumours.

Effects of curcumin on human pituitary tumour cells

To prove the relevance of the findings obtained with rodent pituitary tumour cell lines for human pituitary adenomas, the effect of curcumin on proliferation, apoptosis and hormone production was studied in primary cell cultures of 25 human pituitary adenomas. The results are summarized in Table 1 and representative findings are shown in Fig. 6. Although human pituitary adenoma cells proliferate very slowly in culture in general, curcumin further suppressed the proliferation in 14 out of 17 human pituitary adenomas studied (Table 1; Fig. 6A and B). Measuring DNA fragmentation by cell death ELISA, a significant induction of apoptosis in response to curcumin treatment was detected in six out
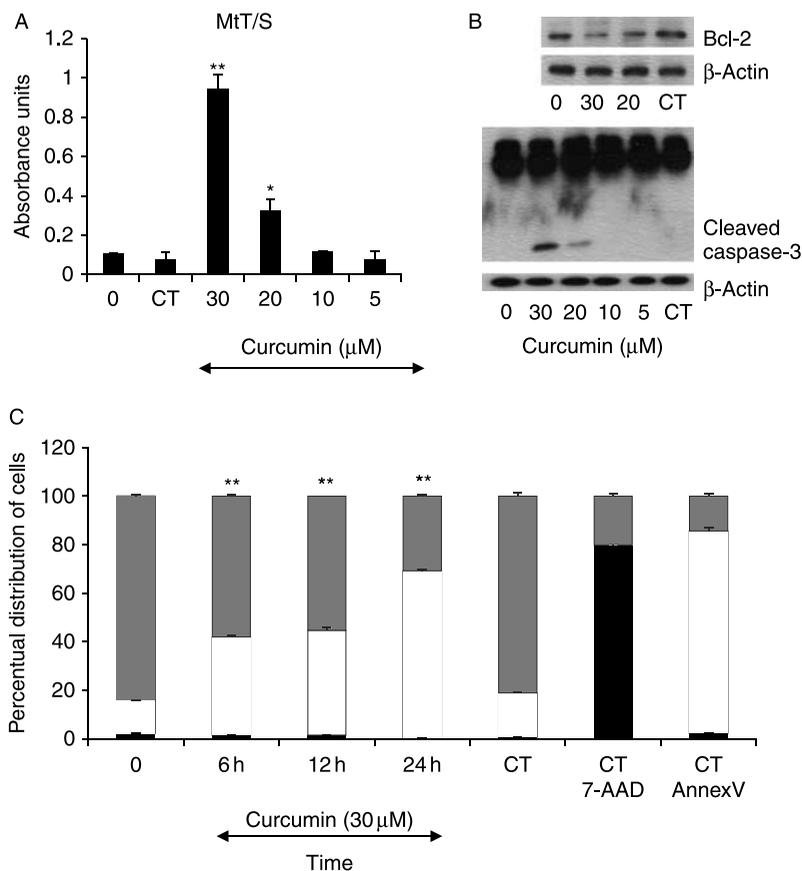
Figure 4 Effect of curcumin on apoptosis and expression of apoptosis-associated proteins in MtT/S cells. (A) Treatment of MtT/S cells with increasing concentrations of curcumin for 24 h induced a dose-dependent, significant increase in DNA fragmentation as measured by cell death ELISA. The application of 30 μM curcumin induced a 9.57 ± 0.71 fold stimulation of apoptosis in comparison to non-treated MtT/S cells. (B) By western immunoblotting, it is shown that 24 h treatment of MtT/S cells with curcumin dose dependently suppressed the expression of anti-apoptotic-acting Bcl-2, whereas pro-apoptotic cleaved caspase-3 expression was enhanced. (C) Detection of normal (grey bars), apoptotic (white bars) and necrotic (black bars) MtT/S cells by FACS analysis after treatment with curcumin (30 μM) for various time periods. Curcumin time dependently and significantly increased the proportion of cells undergoing apoptosis without any sign of induction of necrosis. Non-treated (0) and DMSO-treated (CT) cells were assayed at 24 h and curcumin-treated cells after indicated treatment periods. Positive controls for necrosis (CT 7-AAD) and apoptosis (CT AnnexV) were provided by the manufacturer of the FACS analysis kit. 0, non-treated cells; CT, control with DMSO; $*P < 0.05$; $**P < 0.01$ versus non-treated cells.

of six adenomas studied (Table 1; Fig. 6C). In nine out of 11 endocrine-active human adenomas, hormone production was significantly suppressed by curcumin after stimulation periods of 4 h (not shown) and 24 h (Table 1; Fig. 6D–F). In summary, curcumin acts as anti-proliferative, pro-apoptotic and hormone-suppressive agent in most, but not in all human pituitary adenomas studied.

Discussion

In the present paper, it was demonstrated for the first time that curcumin has anti-tumorigenic actions on rodent and human pituitary tumour cells *in vitro* and *in vivo*. Curcumin inhibited pituitary tumour cell proliferation and induced apoptosis *in vitro* and delayed pituitary tumour progression in an experimental model *in vivo*. Moreover, curcumin reduced hormone production in tumoural pituitary cells. The inhibitory effects of curcumin were not only observed in pituitary tumour cell lines but also to a lesser extent in primary cell cultures of human pituitary adenomas.

Curcumin has been shown to be a potent anti-tumorigenic agent in different types of tumours (Shishodia *et al*. 2007, Hatcher *et al*. 2008). Recently, a first study has demonstrated that curcumin acts in pituitary tumour cells since both growth and hormone production of lactosomatotroph GH3 and lactotroph MMQ cells were suppressed and apoptosis was enhanced by curcumin (Miller *et al*. 2008). Here, it is
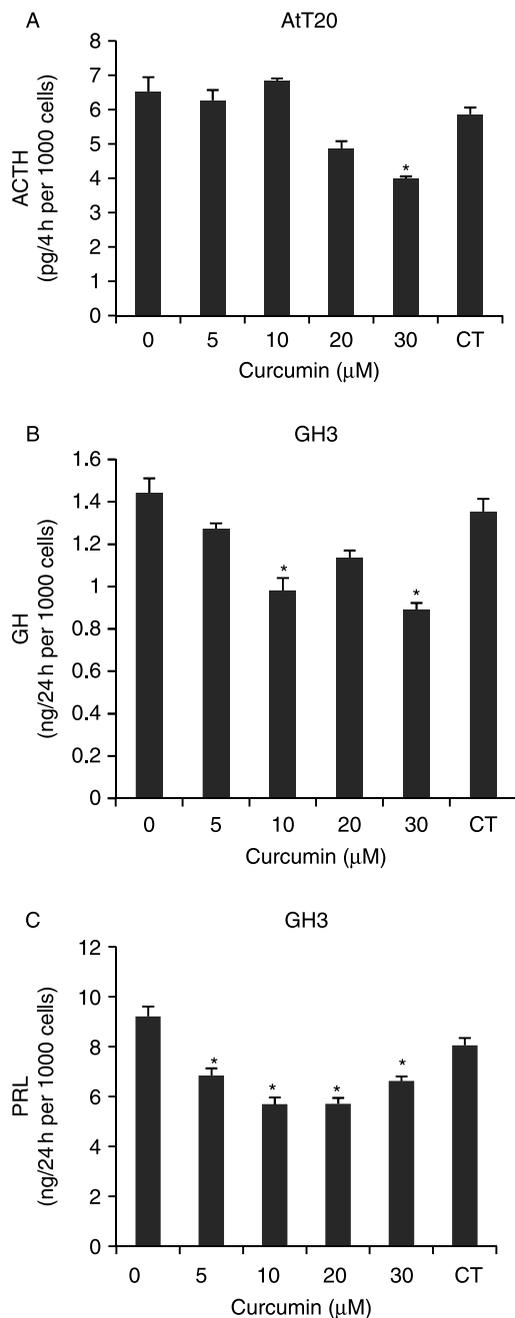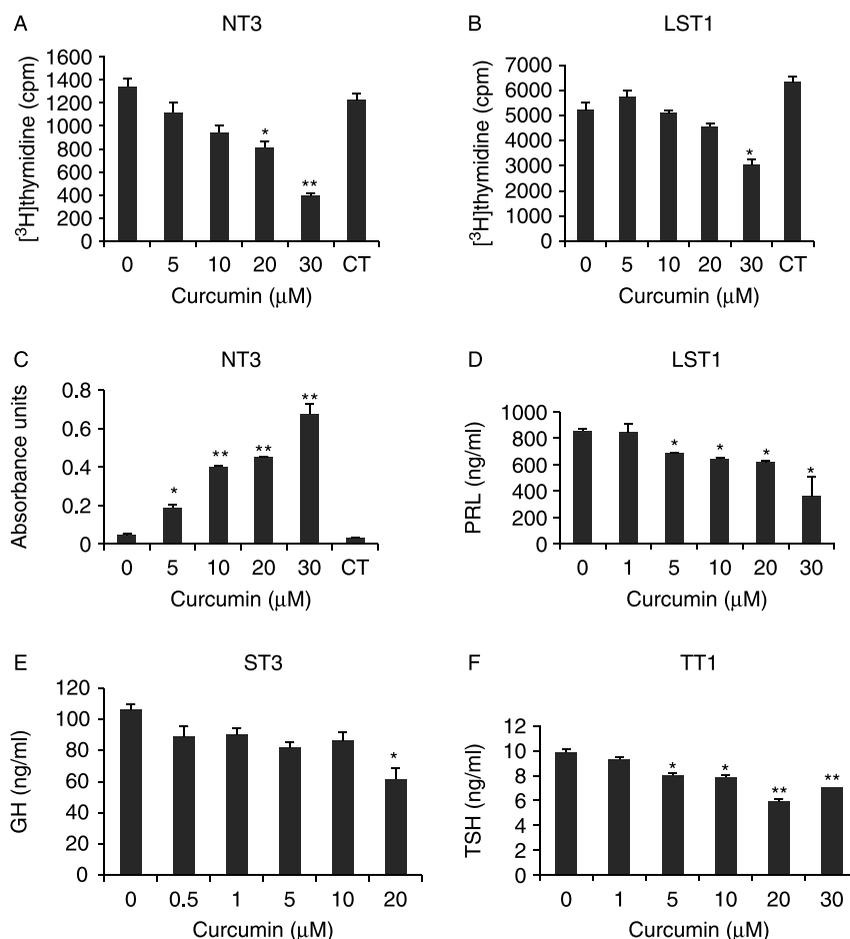
Figure 5 Effect of curcumin on hormone secretion by pituitary tumour cell lines. Corticotroph AtT20 and lactosomatotroph GH3 pituitary tumour cells were stimulated with curcumin (5–30 μM) for 4 and 24 h. At the end of the stimulation period, the cell culture supernatants were cautiously aspirated, centrifuged at 121 **g** to remove floating apoptotic cells, and then used for hormone measurement by RIA. The cells were then trypsinized and counted with an adapted coulter counter to calculate hormone production per cell. In AtT20 cells, ACTH secretion was significantly reduced by curcumin after 4 (A) and 24 (not shown) h, at 20 and 30 μM curcumin concentrations. In GH3 cells, GH and PRL were significantly reduced after 4 (not shown) and 24 (B and C) h. 0, non-treated cells; CT, controls with DMSO; *$P < 0.05$ versus non-treated cells.

shown that these effects occur not only on GH3 cells but also in other pituitary cell lines such as corticotroph AtT20 and somatotroph MtT/S cells. In these cell types, lower concentrations of curcumin as used in the study of Miller *et al*. were sufficient to induce significant inhibition of proliferation. Importantly, using GH3 cells as a representative pituitary tumour cell model, curcumin also induced suppression of tumour growth in a three-dimensional soft agar cell culture model and in experimentally induced GH3 cell tumours in nude mice *in vivo*.

With respect to curcumin's mechanism of action, studies in other types of tumours have shown that curcumin induces cell cycle arrest at G2/M or G1/S phases (Park *et al*. 2006, Srivastava *et al*. 2007). FACS analyses of the pituitary adenoma cell lines studied showed that curcumin treatment led to accumulation in G2/M phase of the cell cycle. This was found to be associated with curcumin-induced inhibition of cell cycle regulators such as cyclin D1 and CDK4, which are critically involved in promoting cells through the initial phase of G1 following G2/M phase. It is likely, but still has to be proven that human pituitary adenoma cells are also arrested in G2/M. However, in contrast to rapidly growing pituitary tumour cell lines, human pituitary adenoma cells in culture grow extremely slowly and therefore, the demonstration of growth inhibition during timely limited treatment periods as well as the determination of the phase of the cell cycle, in which the growth arrest takes place, is difficult. Nevertheless, in most, but not in all human pituitary adenoma cell cultures studied, a curcumin-induced growth suppression of adenoma cells was observed.

Tumour growth arrest is often associated with induction of cell apoptosis, and in a number of different tumour cells, curcumin has been shown to induce apoptosis in parallel with growth inhibition (Cao *et al*. 2007, Dujic *et al*. 2008). Herein, we have also demonstrated that curcumin initiated apoptosis in AtT20, GH3 and MtT/S pituitary tumour cells. Even the highest concentration of curcumin did not induce necrosis in the cell lines studied indicating that curcumin has no non-specific cytotoxic effects on pituitary tumour cells. Regarding the apoptotic mechanisms, suppression of Bcl-2 and enhancement of cleaved caspase-3 were observed in all cell lines investigated. These components are downstream modulators of different pro-apoptotic signalling pathways and therefore, it is not yet clear which of the various apoptotic signalling pathways are affected by curcumin in pituitary tumour cells. Curcumin did also induce apoptosis in six out of six human pituitary adenoma cell cultures investigated. Thus, curcumin

Figure 6 Effect of curcumin on human pituitary adenoma cells *in vitro*. Human pituitary adenoma cell cultures were treated with curcumin (0.5–30 μM) and effects on growth, apoptosis and hormone secretion were measured. An overview about all studies in human pituitary tumour cell cultures is documented in Table 1. The figures presented here show dose-dependent curcumin suppression of [^{3}H]thymidine incorporation in a non-functioning ((A) NT3) and a lactosomatotroph ((B) LST1) pituitary adenoma. Curcumin-induced apoptosis measured by cell death ELISA in a non-functioning adenoma (NT3) is shown in (C). Inhibition of GH, PRL and TSH secretion by curcumin in lactosomatotroph (LST1), somatotroph (ST3) and thyreotroph (TT1) adenomas is shown in (D), (E) and (F) respectively. In all experiments, DMSO had no effect on human pituitary adenoma cells. 0, non-treated cells; CT, controls with DMSO; $*P < 0.05$; $**P < 0.01$ versus basal.

would not only stop expansion of pituitary tumours through its anti-proliferative action but would also induce tumour shrinkage, which is one of the major goals to be achieved by pharmacological treatment of pituitary macroadenomas. However, *in vivo* studies are needed to demonstrate shrinkage of pituitary adenomas during curcumin treatment.

Curcumin only reduced tumour progression but did not completely block GH3 cell tumour expansion in nude mice *in vivo*. One explanation could be that GH3 cell tumours have a high proliferative index and a dense intratumoural vessel system (Perez Castro *et al.* 2003), and thus expand too rapidly to be sufficiently blocked by curcumin. In contrast, in slowly growing human pituitary adenomas, which normally have

a proliferation index below 1% (Ezzat & Asa 2006) and are poorly vascularized, curcumin may more effectively suppress tumour expansion and may even induce tumour shrinkage, but corresponding studies are needed to prove this. Another limitation on the action of curcumin in nude mice may be explained by its poor bioavailability due to its rapid degradation *in vivo*. Similarly as shown here, curcumin has been demonstrated to act anti-tumorigenic in many different types of tumours, but the effective dosages needed seem to be high and can only be achieved *in vitro* (Anand *et al.* 2007). It is difficult or impossible to reach such effective concentrations *in vivo*, due to the rapid degradation and excretion of curcumin in liver and kidney respectively (Anand *et al.* 2007). Therefore, effective dosages of

curcumin may have only transiently been achieved within GH3 tumours bearing nude mice. Recently, efforts have been started to construct curcumin derivatives with higher stability and better bioavailability (Bisht *et al.* 2007, Pae *et al.* 2008), which may allow better treatment of curcumin-sensitive tumours, among them pituitary adenomas, in the future.

Suppression or at best normalization of hormone secretion is the principal goal in endocrine-active microadenomas (corticotroph tumours and microprolactinomas). However, nearly nothing is known about the effect of curcumin on anterior pituitary hormone production. Only recently, suppression of GH and PRL by curcumin has been demonstrated in GH3 and MMQ pituitary tumour cells (Miller *et al.* 2008). Here the suppressive effect on hormone secretion by GH3 cells was confirmed and moreover, an inhibitory effect on ACTH release by AtT20 cells was also shown. Although the mechanism of action through which curcumin influences hormone production is not known, the ability of curcumin to modify histone acetylation/deacetylation might play a role. Recently, histone H4 deacetylation of the POMC locus has been suggested to play a role in the pathophysiology of Cushing's disease (Bilodeau *et al.* 2006) and thus, future studies are needed to clarify whether the inhibitory action of curcumin on hormone synthesis involves histone modification. Curcumin also suppressed PRL, GH and TSH secretion in most endocrine-active human pituitary adenomas in culture suggesting a possible therapeutic role of curcumin in the treatment of hormone excess in patients with endocrine-active pituitary tumours.

In summary, the data of the present work show that curcumin has anti-proliferative, pro-apoptotic and hormone-suppressive actions on pituitary tumour cells. These effects were not only observed in pituitary tumour cell lines *in vitro* and *in vivo* but also, to a lesser extent, in human pituitary adenoma cells. Therefore, curcumin alone or in combination with already used drugs may represent a promising tool for the development of new pharmacological treatment concepts of pituitary tumours.

Declaration of interest

The authors declare that there are no conflicts of interest.

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