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Curcumin Induces Apoptosis in Human Neuroblastoma Cells *via* Inhibition of NFκB

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Abstract. *Background: Metastasised neuroblastoma is a largely incurable neoplasia in children over one year of age using current treatment protocols. After dissemination to the bone, the survival rate is <7%, indicating an urgent need for novel therapeutic regimes. As curcumin (diferuloylmethane) had been shown to exert strong anticancer effects against diverse human malignancies different from neurblastoma, the antiproliferative effect of curcumin on the growth of human neuroblastoma cell lines was tested. Materials and Methods: Proliferation of neuroblastoma cell lines Lan-5, SK-N-SH and Kelly under the treatment of curcumin over a broad concentration range (1x10^{-5} to 1x10^{2} μM) was assessed using XTT cell proliferation assays. Possible induction of apoptosis through curcumin treatment was assessed by detection of DNA fragmentation. To investigate the effect of curcumin on NFκB activation, the protein levels of the NFκB subunit p65 of curcumin-treated cells were compared to untreated cells using Western blots. Results: Curcumin showed a significant dose-dependent antiproliferative effect on all three neuroblastoma cell lines starting at a concentration of 1x10^{-3} μM. The highest concentration of 1x10^{2} μM significantly reduced the viable cell count to 8-48% depending on the cell line. This antiproliferative effect was mediated through an increased induction of apoptosis by inhibition of NFκB, corroborating earlier findings indicating an antiapoptotic effect of NFκB. Conclusion: Our results suggest that curcumin might hold promise in the treatment of patients suffering from neuroblastoma.*

Neuroblastoma represents the third most common malignancy in children and accounts for at least 15% of all childhood cancer deaths (1). Despite advances in existing therapeutic modalities, including surgery, radiotherapy and dose-intensive chemotherapy, the long-term survival for stage 4 disease has remained over the years at less than 15% (2). Hence, improved therapy for neuroblastoma is imperatively needed. In several studies, the antiproliferative effects of curcumin have been demonstrated in a broad range of human malignancies, including breast, prostate and colon cancer, and hepatocellular carcinoma (3). Curcumin (diferuloylmethane) is a major active component of tumeric (*Curcuma longa*) being responsible for the specific flavour and yellow colour of the common spice, curry. The compound has been found to be pharmacologically safe: clinical trials indicated no dose-limiting toxicity when administered at doses up to 8 g/day for 3 months (4). From a molecular point of view, curcumin has been shown to suppress cellular transformation, proliferation, invasion, angiogenesis and metastasis of several tumour cell types through mechanisms not fully understood (5). However, several reports indicate that curcumin provides these antitumour effects through inhibition of NFκB activation. Under normal conditions, NFκB, a heterodimer consisting of a p50 and a p65 subunit, is kept from translocating into the nucleus through binding to its inhibitor IκBα. In response to diverse stimuli, NFκB activation proceeds sequentially through phosphorylation of IκBα, ubiquitination of IκBα and finally degradation of IκBα in the 26S proteasome, leading to translocation from NFκB into the nucleus (6), where NFκB activates transcription of several genes involved in apoptosis, tumorigenesis and inflammation (7). In this study, the antiproliferative effect of curcumin on three different human neuroblastoma cell lines was investigated. Furthermore, whether curcumin inhibits NFκB expression and might therefore induce apoptosis in neuroblastoma cells was investigated.

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Materials and Methods

Reagents. Curcumin used in this study was kindly provided by Professor Dr. J. Greten (Deutsche Gesellschaft für Traditionelle

Chinesische Medizin, Heidelberg, Germany). Curcumin was dissolved in pure ethanol (EtOH) and further diluted with phosphate-buffered saline (PBS) so that the final EtOH concentration never exceeded 1% of the cell culture medium.

Human neuroblastoma cell lines and cell culture. Three different human neuroblastoma cell lines were used, namely Kelly, Lan-5 and SK-N-SH, which were kindly provided by Professor Dr. R. Erttmann (Abteilung Pädiatrische Hämatologie und Onkologie, Universitätklinikum Hamburg-Eppendorf, Deutschland). The cell line Kelly was primarily derived from a 1.1-year-old boy (8), Lan-5 from an 0.4-year-old boy (9) and SK-N-SH from a 4-year-old girl (10). Cell lines were grown as monolayers in culture flasks (Nunc, Roskilde, Denmark) in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin (all obtained from Gibco® Invitrogen, Carlsbad, USA) in a 37˚C humidified 5% CO_2 atmosphere.

Cell proliferation assay. To investigate the effect of curcumin on tumour cell proliferation, neuroblastoma cells were seeded at densities of 10,000 (Kelly), 20,000 (SK-N-SH) or 40,000 cells/ml (Lan-5) into 96-well culture plates (Greiner, Frickenhausen, Germany) and allowed to attach for 48 hours. Cells were then incubated with eight different concentrations of curcumin ranging from $1x10^{-5}$ µM to $1x10^{2}$ µM for 48 hours. Cells were subsequently incubated with a combined solution of the tetrazolium compound sodium 3'-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-*bis* (4-methoxy-6-nitro) benzene sulfonic acid hydrate (XTT) and the electron-coupling reagent PMS (*N*-methyl dibenzopyrazine methyl sulfate) to determine the quantity of formazan product formed by reduction of XTT reagent by viable cells; the absorbance of formazan at 490 nm is directly proportional to the number of viable cells in culture. Absorbance of untreated control cells was taken as 100% survival and absorbance of treated cells was taken as a percentage of the survival of the control. To ascertain statistical differences between the control cells and the treated cells, a Friedman-test followed by Dunn's post-test was performed. $P<0.05$ was considered statistically significant. Furthermore, dose response curves were established and the concentration provoking a 50% response, the EC_{50} value, was calculated. Each concentration was tested in quadruplicates and each experiment was repeated independently three times. All statistical tests were performed using GraphPad Prism software (GraphPad Software Inc., San Diego, CA, USA).

Detection of apoptosis. Induction of apoptosis through curcumin treatment was analysed in the Kelly cell line. Cells were separated *via* trypsinisation and transferred into six-well microtitre plates (Greiner Bio-One GmbH, Frickenhausen, Germany) at a density of $1.5x10^{4}$ cells/200 µl and cultured for 24 hours. New culture medium was added containing 10 or 100 µM of curcumin for the treated cells, or pure medium containing 1.0% EtOH was added for control cells, and cells were incubated for one hour. Thereafter, cytospins were prepared as follows: floating and trypsinated adherent cells were both collected and centrifuged for 10 min at 1500 rpm. The supernatant was decanted, cells were re-suspended in 600 µl PBS and 200 µl of this solution were spun down on glass slides through centrifugation for 3 min at 1500 rpm in a cytocentrifuge. Apoptotic cells on the cytospin preparation were visualised using the Fluorescein-FragEL™DNA Fragmentation Detection Kit (Calbiochem®, Darmstadt, Germany) according to the manufacturer's instructions. As a positive control, HL-60 cells treated with actinomycin D, inducing apoptosis by inhibiting RNA synthesis, were processed the same way. All cells were visualized using DAPI (4,6-diamidino-2-phenylindole) staining. All slides where subsequently analysed for apoptosis using a confocal laser microscope (TCS SP2; Leica, Heidelberg, Germany) fitted with both a DAPI filter (330-380 nm) and a fluorescein filter (465-495 nm).

Western blot analysis. Kelly cells were cultured as described above and allowed to reach 90% confluence. New culture medium was added containing 100 µM of curcumin for the treated cells or pure medium containing 1.0% EtOH for control cells. After incubation for 24 hours, cells were washed twice in ice-cold PBS and harvested in 1 ml PBS using a cell scraper (Greiner). Protein was extracted as described elsewhere (11). Protein concentrations were measured using the Bradford method (12). Forty µg of protein per lane were boiled in sample buffer (12.5% 0.5 M Tris-HCl (pH 6.8), 25% glycerol, 20% SDS, 2% bromphenol blue, 0.5% mercaptoethanol) for four minutes at 95˚C. Proteins were separated using 13% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (Bio-Rad, Hercules, CA, USA). After electrophoresis, the separated proteins were electrotransferred onto a Hybond ECL Nitrocellulose Membran® (Amersham Biosciences, Freiburg, Germany) using a Mini Trans-Blot® Electrophoretic Transfer Cell (Bio-Rad). After blocking with 4% milk powder in PBS containing 0.5% Tween (Sigma, St. Louis, MO, USA) for 30 min, membranes were incubated with an 1:200 diluted mouse anti-human anti-NFκB p65 monoclonal antibody (Santa Cruz, Biotechnology Inc., Santa Cruz, CA, USA) overnight at 4˚C. The membranes were then washed with PBS containing 0.05% Tween (Sigma) and incubated with an 1:500 diluted polyclonal rabbit anti-mouse antibody (DakoCytomation, Glostrup, Denmark) conjugated with horseradish peroxidase for 90 min at room temperature. The bound immune complexes were visualised using the electrochemiluminescence Western blotting detection reagents and analysis system (Amersham Biosciences). All Western blot experiments were performed three times independently.

Results

Curcumin inhibited the cell proliferation of human neuroblastoma cells. The results of the cell proliferation assays are summarised in Figure 1. Curcumin inhibited the cell proliferation of all three human neuroblastoma cell lines in a dose-dependent manner. At a concentration of $1x10^{-3}$ µM curcumin already significantly reduced the percentage of viable Kelly cells to 61% ($p<0.01$), of SK-N-SH cells to 67% ($p<0.001$) and of Lan-5 cells to 54% ($p<0.01$) of that of the control, respectively. At the maximum concentration of $1x10^{2}$ µM, curcumin significantly reduced the percentage of viable Kelly cells to 22%, of SK-N-SH cells to 48% and of Lan-5 cells to 8% of that of the control, respectively (all $p<0.001$). Furthermore, the dose response curves were calculated for each cell line, and

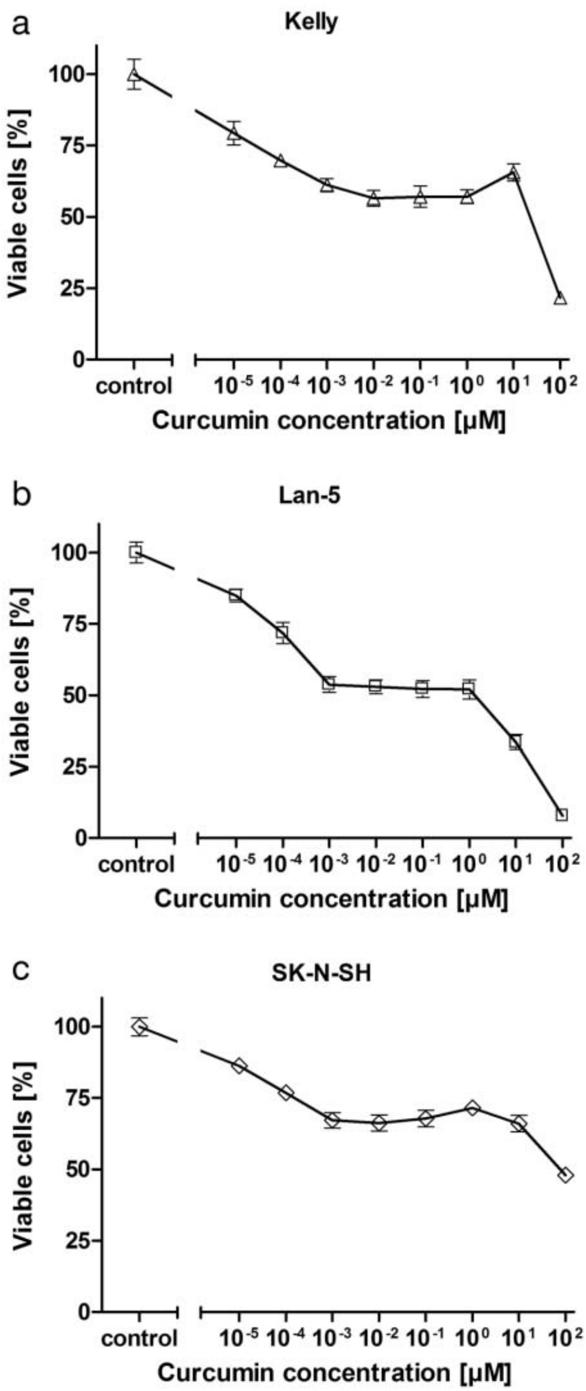

Figure 1. *In vitro effects of curcumin on three different human neuroblastoma cell lines. Neuroblastoma cells were incubated for 48 h with eight different concentrations ranging from 1x10^{-5} µM to 1x10^{2} µM of curcumin and cell proliferation was measured using a XTT-based assay. Values indicate proliferation as a percentage of the respective controls. Curcumin showed a dose-dependent antiproliferative effect on the three neuroblastoma cell lines Kelly (a), Lan-5 (b) and SK-N-SH (c). The maximum curcumin concentration of 1x10^{2} µM significantly reduced the viable cells of Kelly to 22%, of SK-N-SH to 48% and of Lan-5 to 8% that of the control. Error bars represent SD of three independent experiments performed in quadruplicate.*

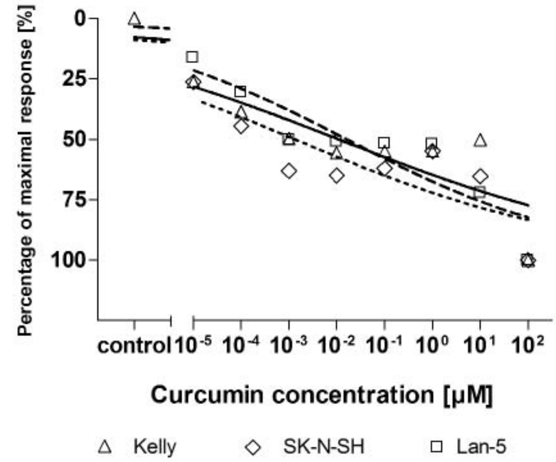

Figure 2. *Dose response curves of curcumin on neuroblastoma cell lines. Dose response curve of Kelly: continuous line, of Lan-5: interrupted line and of SK-N-SH: dotted line. From the dose response curves, the EC_{50} values were calculated as follows, for Kelly: 1.1x10^{-2} µM, for SK-N-SH: 1.3x10^{-3} µM and for Lan-5: 1.6x10^{-2} µM. The EC_{50} value of curcumin for the the cell line SK-N-SH indicates the higher susceptibility of SK-N-SH cells to curcumin in comparision with Kelly or Lan-5 cells.*

showed the typical sigmoidal shape (Figure 2). In addition, the EC_{50} values were calculated as follows: SK-N-SH 1.3x10^{-3} µm, Kelly 1.1x10^{-2} µM and Lan-5 1.6x10^{-2} µM. The EC_{50} values of curcumin for the SK-N-SH cell line was approximately 10-fold lower than for the Kelly and Lan-5 cell lines indicating a higher susceptibility of SK-N-SH to curcumin in comparison to Kelly and Lan-5 cells.

Curcumin-induced apoptosis in human neuroblastoma cells. After curcumin treatment, morphological changes of neuroblastoma cells were observed, namely a significant number of neuroblastoma cells started rounding up and exhibited cell shrinkage, chromatin condensation and nuclear fragmentation typical of apoptotic body formation (13). The results from the apoptosis assay are illustrated in Figure 3. Apoptotic cells were visualized by the Fluorescein-FragEL™DNA Fragmentation Detection Kit and the total cell population was visualized *via* DAPI staining. The proportion of apoptotic cells significantly increased through exposure to 10 µM curcumin (22.4%) and 100 µM curcumin (71.6%), as compared to non-treated control cells (10.3%). The total cell population decreased with these treatments.

Curcumin reduced NFκB protein levels. After treatment with 100 µM curcumin for 24 h, Western blots indicated that curcumin reduced the protein level of the NFκB p65 subunit in Kelly cells, both in the cytoplasmic and in the nuclear protein fractions (Figure 4).

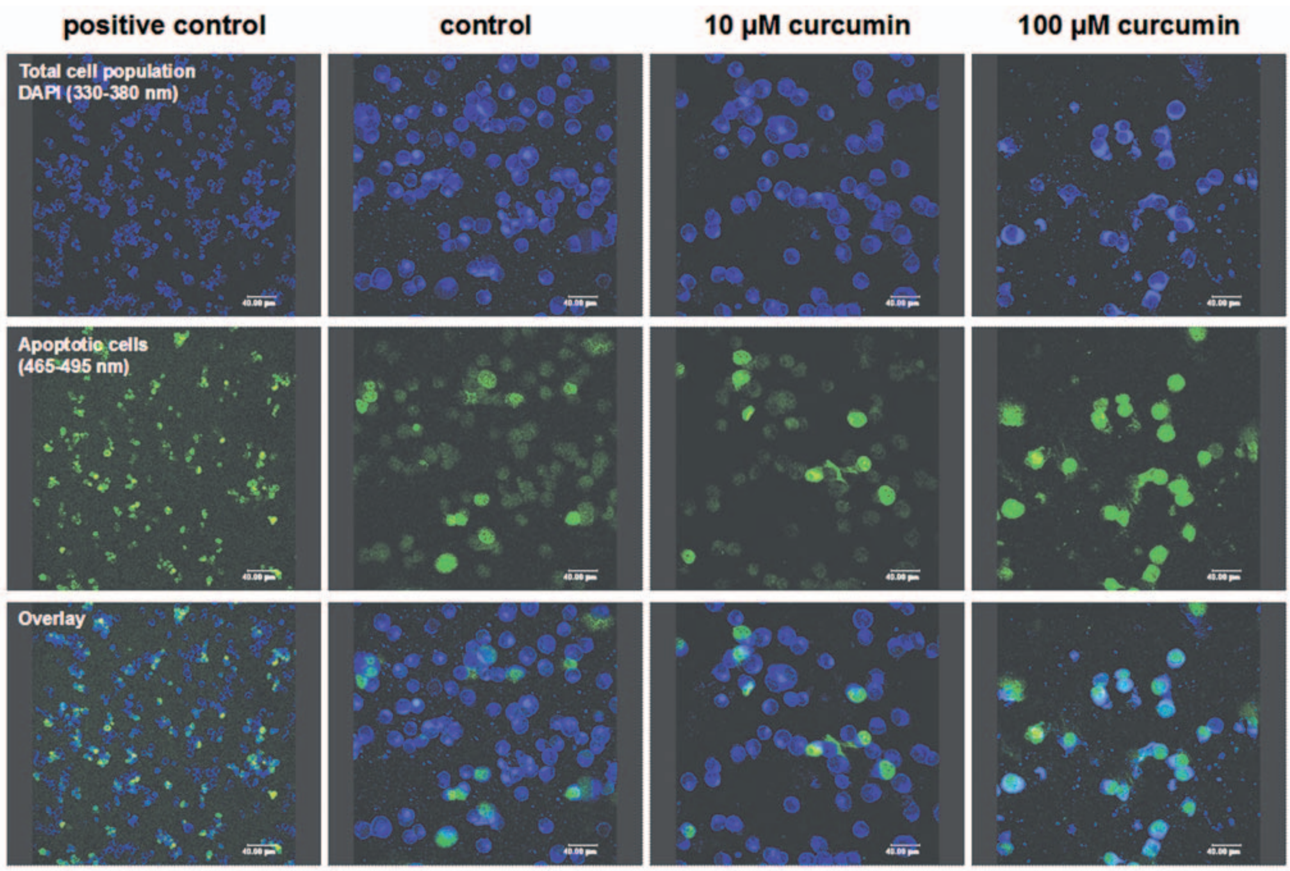

Figure 3. *Detection of apoptosis in curcumin-treated neuroblastoma cells. Kelly cells were incubated for 1 h with 10 μM or 100 μM of curcumin, or with 1.0% EtOH for control cells and apoptotic cells were visualised using the Flourescein-FragEL™DNA Fragmentation Detection Kit. As a positive control, HL-60 cells treated with actinomycin were processed in the same way. The proportion of apoptotic cells significantly increased through exposure to 10 μM curcumin (22.4%) and 100 μM curcumin (71.6%) as compared to non-treated control cells (10.3%).*

Discussion

Despite progress in treatment modalities, many children with advanced neuroblastoma face a poor prognosis and improved therapy is imperatively needed. As curcumin was shown to develop high antiproliferative effects on several human malignancies (3), we tested the effect of curcumin on three different human neuroblastoma cell lines. Furthermore, the effect of curcumin on the apoptosis rate and the NFκB signalling pathway in neuroblastoma cells was investigated. In this study, we demonstrated a significant antiproliferative effect of curcumin on all three neuroblastoma cells using XTT cell proliferation assays. At a concentration of $1x10^{-3}$ μM curcumin already significantly reduced the percentage of viable cells of all three neuroblastoma cells to 54-67% of the control and at the maximum concentration of $1x10^{2}$ μM to 8-48% of the control, respectively. The respective EC_{50} values ranged from $1.3x10^{-3}$ μM to $1.6x10^{-2}$ μM. This suppression of cell proliferation by curcumin is in agreement with those of Bharti *et al.* who showed that 1 μM curcumin inhibited the cell proliferation of human multiple myeloma cells by 23%-51% (14). The decrease in cell proliferation was due to increased apoptosis in neuroblastoma cells induced by curcumin. As demonstrated with a fluorescence assay, treatment with curcumin induced apoptosis in neuroblastoma cells in a dose-dependent manner. These results are in agreement with

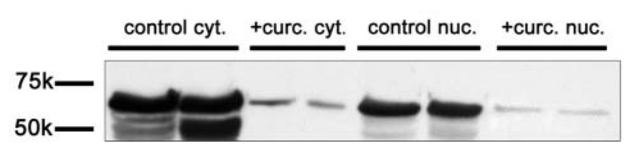

Figure 4. *Cytoplasmic and nuclear NFκB (p65 subunit) protein content in curcumin-treated neuroblastoma cells. Kelly cells were incubated with 100 μM curcumin (+curc.) or 1.0 % EtOH (control cells) for 24 h. Curcumin-treated cells showed a less intensive NFκB p65 subunit protein band both in the cytoplasmic (cyt.) and in the nuclear (nuc.) fractions.*

several reports indicating that antiproliferative effects of curcumin are mediated through increased apoptosis. Ramachandran *et al.* showed that curcumin induced apoptosis in human breast cancer cells through expression of various apoptosis-associated genes (15).

Several lines of evidence suggest that many of curcumin's effects are mediated through the inhibition of NFκB, a transcription factor which regulates expression of various genes with crucial effects on apoptosis, tumorigenesis and inflammation (7). In this study, Western blots were used to show that NFκB is active in neuroblastoma cells as examined with the Kelly cell line. The results are in agreement with two recent reports. Karacay *et al.* showed that SK-N-SH neuroblastoma cells express a high level of NFκB activity using a luciferase reporter gene (16) and Bian *et al.* investigated the crucial role of NFκB in the survival of S-type neuroblastoma cells (17). As NFκB activation is responsible for the transcription of antiapoptotic prosurvival factors such as Bcl-2, FLIP and Akt (2), these mechanisms may contribute to the observed clinical resistance of neuroblastoma tumours to existing therapeutic modalities. In addition, we demonstrated that curcumin suppressed constitutive NFκB activation in Kelly cells. Western blots showed that curcumin highly reduced the levels of the NFκB p65 subunit, both in the cytoplasmic and in the nuclear protein fractions. These results are in agreement with previous reports that curcumin is a potent inhibitor of NFκB activation. Lee *et al.* showed that curcumin inhibited interferon-α-induced NFκB expression in human lung cancer A549 cells (18). The exact molecular mechanism by which curcumin suppresses NFκB activation is not fully understood and needs to be further explored. Furthermore, Bharti *et al.* demonstrated that treatment with curcumin sensitised multiple myeloma cells to cytotoxic agents, such as vincristine and melphalan (14). As NFκB seems to be implicated in the chemoresistance of tumour cells, the combination of curcumin with conventional chemotherapy should be further explored in neuroblastoma cells.

Non-specific drug toxicity is one of the major problems in drug development. Numerous studies have shown that curcumin is pharmalogically safe. It was recently shown in Phase I clinical trials that humans can tolerate up to 8 g curcumin per day with virtually no deleterious side-effects when it is taken orally for three months with a respective average peak serum concentration of 1.77 ± 1.87 μM (4). Our results suggest the concentration of curcumin that inhibited the cell proliferation of all three neuroblastoma cell lines in a significant manner are two to three orders of magnitude within the range of physiologically achievable serum concentrations.

In conclusion, our results indicate that curcumin, being a pharmacologically safe agent, has high antiproliferative effects on human neuroblastoma cells *in vitro*. This decrease in cell proliferation was due to an increased apoptosis rate in neuroblastoma cells which seemed to be mediated through an inhibtion of the NFκB signalling pathway. Our study provides sufficient evidence for considering curcumin as a potential therapy for patients with neuroblastoma and should therefore be further investigated in clinical trials.

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