Analysis of the Tumoricidal and Anti-Cachectic Potential of Curcumin

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Abstract. *Curcumin, the extract of the rhizome of Curcuma longa, is known for its health-promoting properties in traditional medicine. It has anti-inflammatory, antitumor and antioxidant properties and stimulates appetite. In the present study, we investigated the stability of curcumin and its effect on cytotoxicity, apoptosis and melanin content in melanoma cells and the effect on atrophic C2C12 muscle cells. Cytotoxicity of curcumin was dose-dependent and the EC_{50} for 24-h incubation was 69 μM. Saturation was reached at 30 μM for a 48-h incubation. The EC_{50} for 24-h incubation with degraded curcumin solution was 116 μM and that for 48-h was 94 μM. Curcumin induced a strong increase in caspase-3/7 activity at 30-40 μM. Electrical impedance measurements showed that sub-toxic doses of curcumin counteracted atrophy in an in vitro model system. These findings indicate not only the positive effects of curcumin on melanoma cells in vitro, but also that curcumin was able to considerably trigger anti-cachectic effects in vitro. However, the importance of the stability of curcumin and its tumoricidal and anti-cachectic potential might play a pivotal role in its use in the nutrition and health industrie since it degrades rapidly in aqueous solutions.*

For centuries herbal plants have been used to prevent or cure diseases. Their use is based on the experience of many generations and is part of traditional medicine. However, in modern medicine they are neglected because of the lack of scientific evidence. Only a handful of plants have gained the attention of scientists so far. One of them is *Curcuma longa*. Its rhizome is used as a spice in curry and as a cheap substitute for saffron. In traditional medicine, it is used against a wide spectrum of diseases (1). The main component responsible for its medicinal effects is curcumin, the yellow pigment isolated from the rhizomes. It has anti-oxidant, anti-inflammatory, anti-aggregation and antitumor activities (2-6). It also has protective effects on liver cells after ischemia induction and increases neuronal survival (7, 8). Curcumin also has anticancer properties that are due to induction of apoptosis and cell-cycle arrest (9-12). However, curcumin is unstable in buffer and aqueous solutions and is degraded (Figure 1) to *trans*-6-(4'-hydroxy-3'-methoxyphenyl)-2,4-dioxo-5-hexenal, vanillin, ferulic acid, feruloyl methane (13). Curcumin has a poor bioavailability because of its hydrophobic structure, while its degradation products have greater aqueous solubility. These products might contribute to the large range of pharmacological effects of curcumin (14-21). The bioavailability of curcumin can be increased by concomitant administration of piperin, an extract of black pepper (22).

In addition to the tumoricidal effects of curcumin, its anti-cachectic properties are also of great importance in cancer care. Apart from pain, fatigue, hair loss and other physical and psychological concomitants, loss of weight, also known as cachexia, is a severe consequence for patients with cancer which is caused by chemotherapy, surgery and radiotherapy (23). Cachexia (from the Greek kakos "bad" and hexis "condition") is often described as emaciation, wasting, weight loss and progressive change in vital body functions (24). This condition is often accompanied by loss of appetite (25). The severity of cachexia is dependent on tumor entity. A weight loss was observed in 30 to 80% of patients with cancer (26). Cachexia occurs most frequently in patients who suffer from pancreatic and stomach cancer while, those suffering from breast cancer, non-Hodgkin's lymphomas and sarcomas are affected less often (27). Weight loss is an important prognostic factor – the larger its extent, the lower the survival rate (26).

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Key Words: Curcumin, cytotoxicity, cachexia, B16, C2C12.

Current research deals with the development of agents against the devastating impacts of cachexia. Investigations are carried out *in vitro* as well as *in vitro* and are based on different models. Eley *et al*. showed that treatment with dsRNA-dependent protein kinase inhibitors of cachectic mice which were transplanted subcutaneously with fragments of a murine adenocarcinoma 16 (MAC16) inhibited tumor growth and reduced weight loss (28). Furthermore, they showed by western blot that these inhibitors reduce the activity of the 20S proteasome which is responsible for protein degradation (28). Similar results were obtained by Smith *et al*. who found that β-hydroxy-β-methylbutyrate, a metabolite of leucine, inhibits protein degradation and stimulates protein synthesis in skeletal muscles (29).

In another *in vitro* model for cachexia, the increase of interleukin-6 (IL6) was detected in mice which were transplanted with the cell line C-26.IVX (30). Within the same study, it was shown that the administration of a monoclonal antibody against mouse IL6 leads to inhibition of IL6 activity and hence to a counterattack against cachexia (30). Springer *et al*. induced cachexia by injection of hepatoma cells into the peritoneal cavity of rats (31).

Several signaling pathways in cancer-induced cachexia which target the ubiquitin-proteasome system were identified. For example, myostatin in its cleaved and activated form regulates nuclear factor κ-light chain-enhancer of activated B-cells (NFκB)-independent signaling pathway through its binding to type II serine/threonine kinase receptors (32). The expression of muscle atrophy F-box which is a gene coding for an E3 ubiquitin ligase is up-regulated (32). By proteolysis inducing factor and other cytokines, NFκB is released *via* the IκB kinase complex and moves to the nucleus where it regulates the expression of genes coding for E3 ubiquitin ligases (32).

In view of these facts, we analysed the effects of curcumin to determine its potential as an anticancer drug. Additionally, anti-cachectic/hypertrophic properties of curcumin were also investigated *in vitro*.

Materials and Methods

Cells and cell cultures. Murine melanoma B16-F1 cells (CLS, Eppelheim, Germany) (were cultured in RPMI-1640 supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin (all Sigma-Aldrich, Steinheim, Germany) and incubated at 37˚C in 5% CO_2. Curcumin (Sigma-Aldrich, Steinheim, Germany) was dissolved in ethanol (70%) at a concentration of 10 mM and was diluted to the required concentration with Quantum 263 medium (PAA, Pasching, Austria) immediately before use. Cells grown in medium containing an equivalent final volume of ethanol served as a control.

C2C12 myoblasts (CRL-1772, ATCC, Manassas, VA, USA) were cultured in RPMI-1640 supplemented with 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin and incubated at 37˚C in 5% CO_2. For differentiation into myotubes, culture medium was exchanged with RPMI-1640 containing 2% horse serum, 1% ITS Liquid Media Supplement (Sigma-Aldrich, Steinheim, Germany), 100 U/ml penicillin and 100 μg/ml streptomycin. For impedance experiments, C2C12 myoblasts were plated on an electrode-containing plate (E-Plate 96; Roche Applied Science, Indianapolis, IN, USA) which was pre-treated with 0.1% gelatine solution and differentiated using the differentiation media mentioned above for 5, 7 and 10 days, respectively. C2C12 cells were plated at a density of 5×10^3 per well and differentiated as described above. Myotubes were treated for 24 h with 100 μM dexamethasone for atrophy induction and subsequently with different concentrations of curcumin for an additional 24 h.

Stability of curcumin in cell culture medium. The loss of curcumin because of degradation was determined by measuring UV/VIS spectra. Samples of freshly prepared or 24-h-old curcumin solutions or cell supernatant after 24-h incubation were measured from 220 nm to 750 nm.

Cytotoxicity analysis (LDH release assay). B16-F1 cells were plated in 96-multiwell-plates at a density of 1×10^4 cells/well and cultivated for 2 h before incubation with 200 μl of Quantum 263 Medium (PAA) containing final concentrations of 20-150 μM curcumin (Sigma-Aldrich) for 24 and 48 h. After these incubations, the lactate dehydrogenase (LDH) activity in 100-μl cell culture supernatant was measured with an LDH release assay (Cytotoxicity Detection Kit (Roche, Mannheim, Germany)). Relative cell death was determined as the percentage of LDH activity divided by the maximum amount of releasable LDH activity by lysing the cells of the positive control with Triton X-100 (final concentration: 1% Triton X-100).

Cytotoxicity analysis (WST-1 assay). The cytotoxicity of curcumin was tested by WST-1 assay (Cell Proliferation Reagent WST-1; Roche) using the murine melanoma cell line B16-F1, murine C2C12 myoblasts (non-differentiated) and murine C2C12 myotubes (terminally differentiated).

The B16-F1 cells were plated in 96-multiwell-plates at a density of 1×10^4 cells/well and C2C12 cells were plated at a density of 5×10^3 cells/well in a volume of 0.15 ml, respectively. Cells were cultivated for 24 h before incubation with culture medium containing final concentrations of 10-50 μM curcumin for an additional 24 h. Cells were either treated with different concentrations of curcumin or remained untreated as a control. Subsequently, 15 μl WST-1 assay reagent (Cell Proliferation Reagent WST-1; Roche) was added to each well and cells incubated for 2 h. The absorbance was determined using a microplate reader at 450 nm against a reference wavelength of 690 nm. Half-maximal effective concentrations (EC_{50}) for each cell line were evaluated, representing the concentration at which cytotoxicity was 50%. To calculate the EC_{50} value for curcumin, non-linear statistical regression analysis was performed using SigmaPlot (Version 11.0; Systat Software GmbH, Erkrath, Germany).

Apoptosis induction. Apoptosis induction was measured with a luminescent assay. B16-F1 cells were plated in 96-multiwell-plates at a density of 1×10^4 cells/well and cultivated for 2 h before incubation with 200 μl of Quantum 263 Medium (PAA) containing final concentrations of 20-80 μM curcumin (Sigma-Aldrich) for 24 h. Then the caspase-3/7 activity was measured using Caspase-Glo® 3/7 Assay (Promega, Madison, WI, USA).

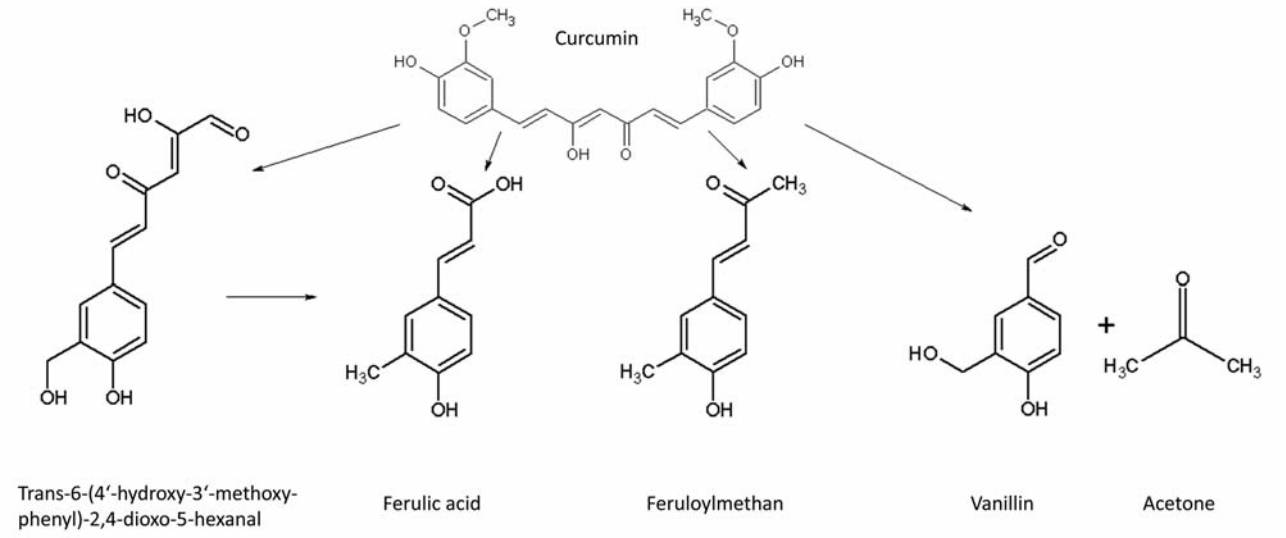

Figure 1. *Structure of curcumin and its degradation products.*

Melanin content measurement. B16-F1 cells were plated in 12-multiwell plates at a density of 5×10^4 cells using Quantum 263 medium. The cells were treated with curcumin for 24 h and 48 h, respectively. Then the supernatant was discarded and the monolayer was washed twice with PBS.For the positive control, cells were lysed with 100 µl of 2% Triton-X. The absorbance was measured at 450 nm using a microplate reader. The results are expressed as a percentage of the positive control.

Electric impedance measurement. Impedance measurements were taken by the xCELLigence RTCA System (Real-Time Cell Analyzer; Roche Applied Science) using an E-Plates 96-well device. This system was used in a commercially-available cell incubator at 37˚C and 5% CO_2. C2C12 cells were trypsinized and seeded on a 96-well E-Plate pre-coated with 0.1% gelatine solution at a density of 5×10^3 cells per well in a volume of 0.15 ml. Cells were incubated at room temperature for 30 min to ensure uniform sedimentation of the cells and then put back into the system. Differentiation was maintained for 5, 7 and 10 days respectively and was carried out by exchanging the culture medium every 24 hours with differentiation medium. Dexamethasone (atrophy stimulation) or curcumin (hypertrophy stimulation) was also applied by media exchange. xCELLigence RTCA System software (version 1.0, Roche Applied Science) was set such that the cell index value (which corresponds to electrical impedance) was measured every 15 min during C2C12 differentiation and induction of atrophy/hypertrophy. The resulting data were normalized to the impedance value for each well immediately prior to curcumin addition using the same software.

Results

Curcumin and its degradation products induce cell death in melanoma cells. Firstly, we investigated the cytotoxicity of curcumin and its degradation products (Figure 1) on B16-F1 melanoma cells in monolayer cultures. Figure 2 shows the results. The effect of curcumin on B16-F1 cell death increased in a dose-dependent manner. For 24-h incubation with curcumin, the EC_{50} was approximately 69 µM. An EC_{50} value for 48-h incubation with curcumin could not be determined because cytotoxicity did not exceed 30%.

Because of its rapid degradation in aqueous solutions, we also investigated the cytotoxicity of degraded curcumin. Incubation for 24 h and 48 h with degraded curcumin slightly reduced cell cytotoxicity, as shown in Figure 2. The EC_{50} values were approximately 116 µM for 24-h incubation and 94 µM for 48-h incubation, respectively.

Cell death was induced by caspase activation. Figure 3 shows the dose-dependent effect on caspase activation of B16-F1 melanoma cells after 24-h incubation with curcumin. The highest activation yields were reached with 30-40 µM curcumin with 6.1±2.0 and 7.1±1.4 fold activation, respectively.

Incubation with degraded curcumin did not lead to an increase in caspase 3/7 activity (data not shown).

Curcumin decreased melanin content in melanoma cells. Melanin content in B16-F1 cells was measured after curcumin incubation. Curcumin showed no cytotoxic effect on B16-F1 cells at concentrations less than 20 µM but reduced the melanin content in a dose-dependent manner (Figure 4). The strongest decrease was reached at 5 µM curcumin.

Determination of sub-lethal concentrations of curcumin on C2C12 and B16-F1 cells. To calculate the EC_{50} value for curcumin, non-linear statistical regression analysis was performed. Figure 4 describes the cytotoxicity of curcumin on non-differentiated and terminally-differentiated C2C12 cells.

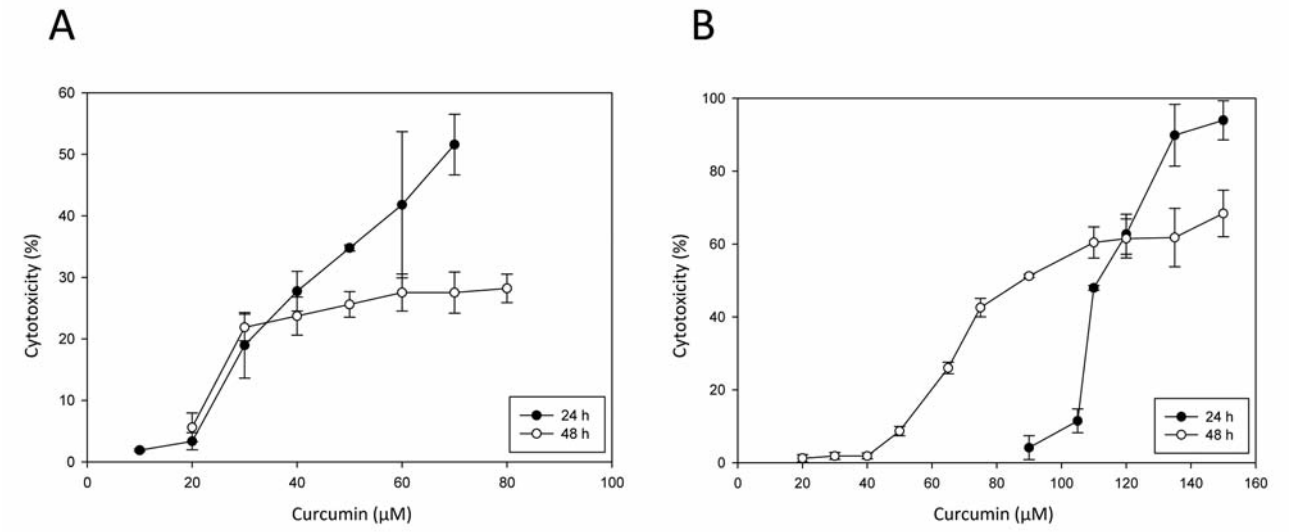

Figure 2. *A: Cytotoxicity of fresh curcumin solution. B16-F1 cells were incubated with different curcumin concentrations for 24 h and 48 h, respectively. Cytotoxicity was determined with a lactate-dehydrogenase release assay (LDH). The EC_{50} value for the 24 h incubation with fresh curcumin solution was 68.8±1.2 μM. B: Cytotoxicity of degradation products of curcumin. B16-F1 cells were incubated with different concentrations of a 24-h-old curcumin solution for 24 h and 48 h, respectively. The EC_{50} value for the 24 h incubation with curcumin solution was 115.8±1.5 μM and for 48 h was 94.1±3.4 μM.*

B16-F1 cells were treated with curcumin for 24 h. Subsequently WST-1 assay was performed to determine cytotoxicity. The EC_{50} value of 23.7 μM for B16-F1 cells was calculated on the basis of a non-linear statistical regression analysis (data not shown). Direct comparison of the results can be seen in Table I.

Table I. *EC_{50} values for curcumin on non-differentiated and terminally differentiated C2C12 cells and B16-F1 murine melanoma cells.*

Cell line	EC_{50} (μM)
C2C12 Non-differentiated	19
C2C12 Differentiated	18.4
B16-F1 (melanoma cells)	23.7

Induction of atrophy and its inhibition by curcumin treatment. As described by Rakhilin *et al.*, dexamethasone was used to induce atrophy (33). The atrophy model described here made use of dexamethasone for the same purpose. The results of all performed investigations and the effects of curcumin were conducted with atrophic C2C12 myotubes.

Furthermore, correlations between normalized cell index values (CI) and morphological changes of the cells were created. Normalization of CI values took place at the time of atrophy induction (data not shown) and at the time of curcumin treatment.

Sub-lethal concentrations of curcumin counteracted atrophy in an *in vitro* model system.

CI values increased in all wells treated with curcumin. The lowest concentration (5 μM) caused no further increase in CI value after 5 h. Interestingly, the highest concentration of curcumin (20 μM) caused the highest CI. The overall increase in CI during curcumin treatment was 20% (Figure 5A).

Due to treatment of cells with different concentrations of curcumin, the CI value rose by 20-30%. As an exception, when cells were treated with 5 μM of curcumin, the CI value was relatively constant but after 17 h, CI decreased virtually by 20%. The highest CI value was reached after the addition of 15 μM curcumin (Figure 5B).

CI values increased after treatment with 5 μM curcumin by 60%. Twice the concentration caused the opposite effect. After treatment of atrophic C2C12 myotubes with 10 μM of curcumin, CI values fell directly after a decrease of 40% to a minimum. From a concentration of 15 μM, CI values remained stable but with a downward tendency after 305 h (Figure 5C).

Discussion

Herbal plants have been used to prevent or cure diseases for centuries. The rhizome of *Curcuma longa* is not only used as a spice or colouring agent, but it is used to treat inflammatory diseases (1). The main component providing the medicinal effects is curcumin, the yellow pigment of the rhizome (1). It has a wide spectrum of medicinal effects, *e.g.*

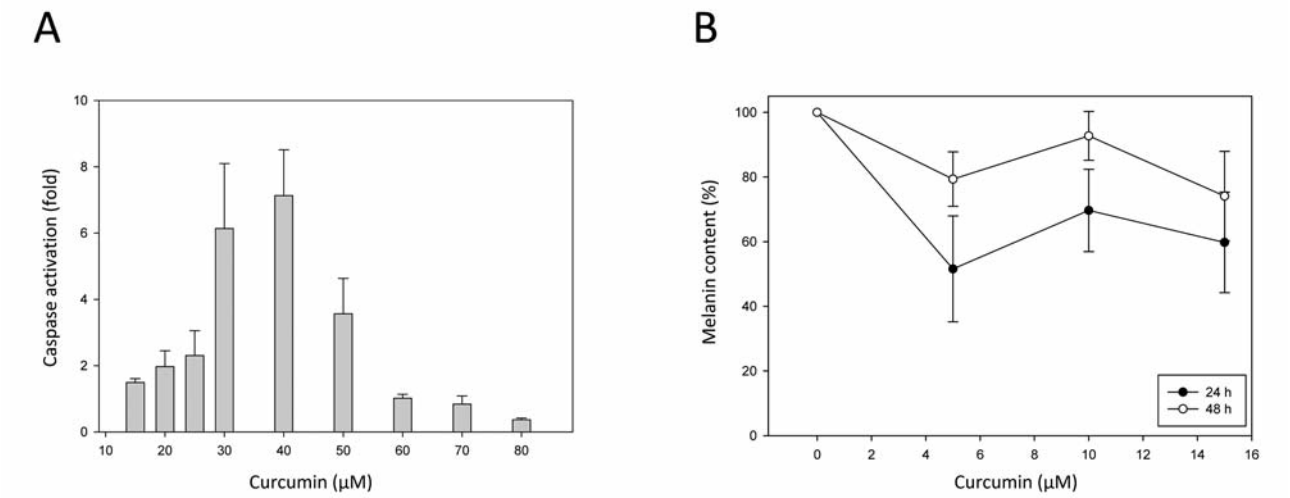

Figure 3. *A: Activation of caspases 3/7 by curcumin. B16-F1 cells were incubated with different curcumin concentrations for 24 h and then the activity of caspases 3 and 7 were determined by a luminescence assay. B: Melanin content reduction by curcumin. B16-F1 cells were incubated with subtoxic concentrations of curcumin for 24 h and 48 h, respectively. Cells were then lysed and absorption at 450 nm was measured.*

anticancer (9-12) or immunomodulatory properties (2-6).

We recognized the importance of further experimentation to gain insight into the influence of curcumin in the field of skin cancer. Malignant melanoma has become the most dangerous type of skin cancer due to its aggressive propagation and resistance to modern therapies and therefore, additional research to solve these problems and discover an optimal therapy is warranted. Past reviews have analyzed and documented the tumoricidal effects of curcumin on a number of melanoma cell lines (34). These studies have identified that the effect of curcumin was due to the inhibition of glutathione-S-transferase activity, the inhibition of cyclooxygenase-1 (COX1) and COX2 enzymes, the induction of apoptosis through the FAS receptor/caspase-8 pathway and the down-regulation of the NFκB pathway. Additional research also showed that the anti-metastatic action of curcumin in melanoma cells was caused not only by the modulation of integrin receptors and the activation of collagenase, but also the expression of non-metastatic gene 23 (NM23) and E-cadherin, the down-regulation of focal adhesion kinase (FAK), and the reduction of matrix metallopeptidase-2 (MMP2) activity. Furthermore, the chemopreventive effects of curcumin on several carcinogen-induced skin cancer models were investigated. Here, curcumin was shown to act as a tool to reverse the resistance of melanoma cells to multiple drugs through the inhibition of glutathione-S-transferases (34).

In the present study, we demonstrated that curcumin is cytotoxic to murine melanoma cells at micromolar concentrations. This cytotoxicity was due to the effect of curcumin on apoptosis activation at lower concentrations. However, the highest apoptosis activation appeared at 30-40 µM, while the EC_{50} value for cytotoxicity was about 68.8±1.2 µM curcumin for a 24 h incubation. At higher curcumin concentrations, the cells might go into necrosis directly, which might lead to an inflammatory reaction. These results show that curcumin only has a small therapeutic window. The EC_{50} value for a 48-h incubation was higher because curcumin degraded rapidly in aqueous solutions as shown elsewhere (13). However, the results indicated that the degradation products still have cytotoxic effects on melanoma cells. Also, an increase in apoptosis activation was not observed. Hence, curcumin could be used as an anticancer therapy but using pre-mixed liquids with curcumin is of no use because of its instability in aqueous solutions.

When administered at very low concentrations below cytotoxicity, *i.e.* as an ointment, curcumin is able to reduce pigmentation of melanocytes. The effect might be mediated by the inhibition of tyrosinase related protein 2, an enzyme that is part of the pathway of melanogenesis (35, 36). This feature can be applicable in cosmetics to reduce hyperpigmentation.

In conclusion, curcumin had strong cytotoxic effects on melanoma cells but this application needs to be well-researched to determine the most effective blood serum concentration and to avoid early degradation of curcumin. Of course, further studies are required to confirm the safety and efficacy of curcumin for its clinical use.

Cytotoxicity of curcumin on C2C12 myoblasts and B16-F1 melanoma cells. By WST-1 assays different concentrations of curcumin were tested on C2C12 cells. These cytotoxicity

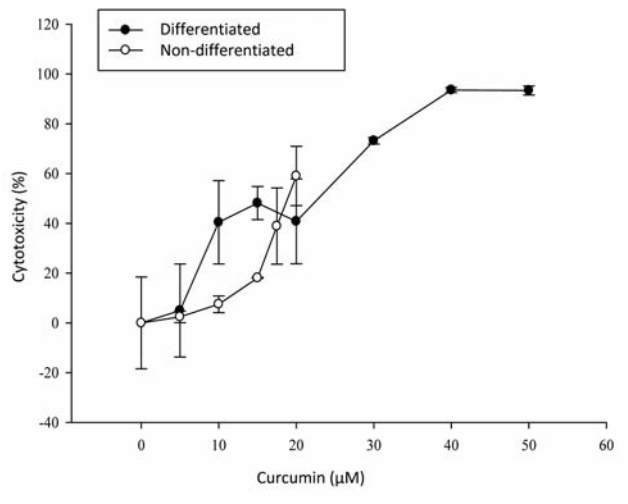

Figure 4. *Cytotoxicity of curcumin towards non-differentiated and terminally differentiated C2C12 cells.*

assays should provide information about EC_{50} values in order to perform stimulation of atrophic cells with sub-lethal concentrations of curcumin. Non-linear statistical regression analysis was suitable for determining the EC_{50} value of curcumin. For non-differentiated C2C12 myoblasts an EC_{50} of 19 μM curcumin and for terminally-differentiated C2C12 myotubes an EC_{50} of 18.4 μM curcumin was determined. Results indicated that tolerance to curcumin was not significantly different between both cell types. The EC_{50} value for B16-F1 melanoma cells was at 23.7 μM on an equal level. Our analysis resulting in a value greater than all mentioned EC50 values indicates an inconsistency with existing literature (36). In order to investigate the regulatory effect of curcumin on melanomas *in vitro*, concentrations should be specified above this limit. For that reason an effect on healthy tissue is not excludable.

Curcumin as an anti-cachectic remedy. For inhibition of atrophy, cells were treated with different concentrations of curcumin. The effect of this substance was regularly observed *via* the xCELLigence system and additionally checked after 24 h by microscopy. While 5 μM curcumin on the fifth and seventh day of differentiation caused a decrease in CI, 5 μM after the tenth day of differentiation caused an increase in CI by 60%.

Specifically, the positive effect of curcumin was found to be a result of its ability to suppress the activity of the transcription factor NFκB by hindering the phosphorylation of the NFκB inhibitor IκB. Normally the inhibitory subunit IκB is bound to the p50/p65 heterodimer of NFκB. But upon stimulation, a specific kinase is activated which leads to phosphorylation of IκB and dissociation from the NFκB

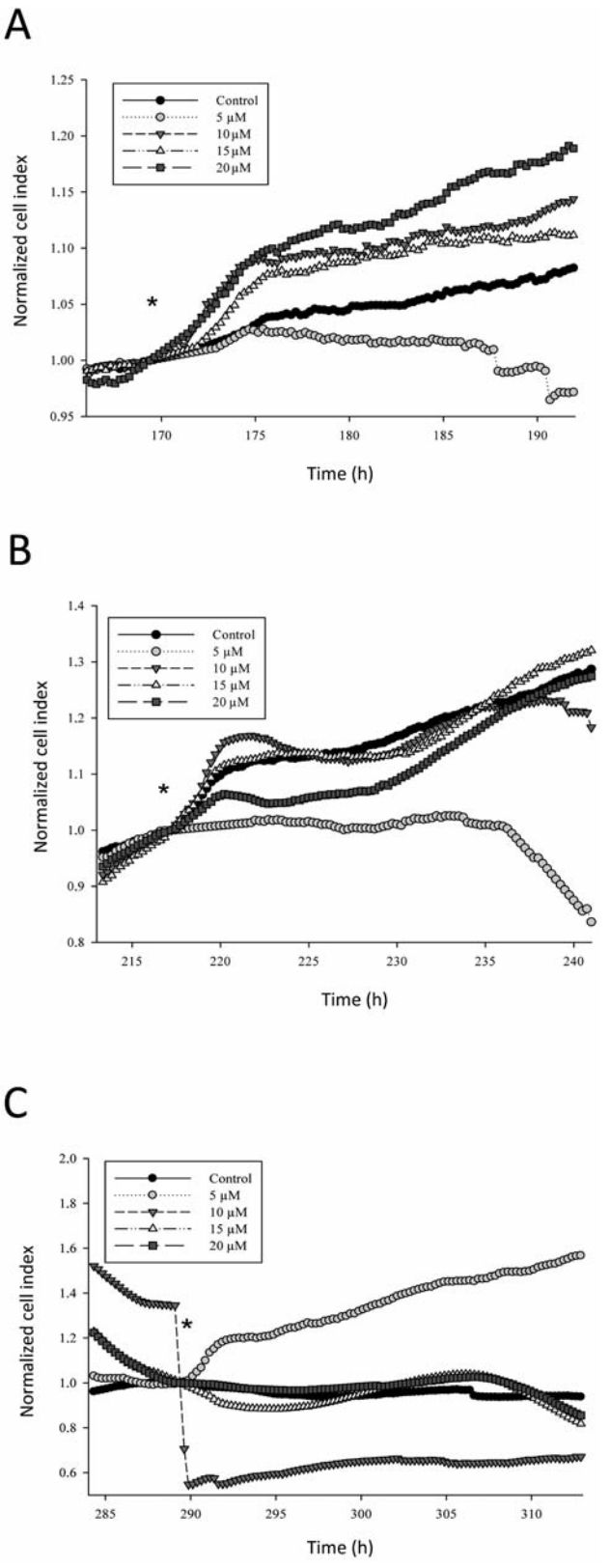

Figure 5. *Normalized CI after the addition (*) of curcumin after five (A), seven (B) and 10 (C) days of differentiation.*

complex (38). Furthermore, the argumentation for treating cachectic tumor-bearing animals with curcumin was supported with the belief that that muscle-wasting is triggered by the tumor necrosis factor-α, along with other mediators. Studies indicated the stimulation of muscle protein degradation through tumor necrosis factor-α during cancer cachexia (38).

Curcumin bioavailability and stability. Animal studies have revealed that curcumin has poor bioavailability due to its rapid metabolism in the liver and the intestinal wall. Shobal *et al*. examined the effect of combining piperine, a known inhibitor of hepatic and intestinal glucuronidation, on the bioavailability of curcumin in rats and healthy human volunteers. This study showed that curcumin when given at a dose of 2 g/kg to rats, without any additional elements, resulted only in a moderate serum concentration increase in a 4-h time period (22). Curcumin along with the additional administration of piperine 20 mg/kg led to an increase in the serum concentration of curcumin for a short time period of 1-2 h (39).

The investigation of curcuminoid stability in physiological media recognized the rapid decomposition (over 90% within 12-h) when serum was omitted and increased stability in the presence of serum. Several degradation products were detected, most of which have not yet been identified (40). *Trans*-6-(4'-hydroxy-3'-methoxy-phenyl)-2,4-dioxo-5-hexenal was predicted as the major degradation product and vanillin, ferulic acid, and feruloyl methane were identified as minor degradation products of short-term reaction (13).

Research is still warranted to identify effective micro-encapsulation of curcumin to enhance its stability in pre-mixed aqueous solutions for patients. However, problems occurring due to instability of curcumin and bioavailability necessitate further studies to discover the optimum pharmacological active concentrations required for successful patient therapy.

Acknowledgements

The work presented here was made possible by funding from the Federal Ministry of Economics and Energy (03EGSSN113, 01.0312-28.02.13)

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Received May 8, 2014
Revised July 15, 2014
Accepted July 16, 2014