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Curcumin in combination with visible light inhibits tumor growth in a xenograft tumor model

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It is known that curcumin, a dietary pigment from the plant *Curcuma longa*, inhibits cell proliferation and induces apoptosis in different cell lines; however, the therapeutic benefit is hampered by very low absorption after transdermal or oral application. Recent studies from our laboratory have demonstrated that curcumin at low concentrations (0.2–1 μg/ml) offered the described effects only when applied with UVA or visible light. Nevertheless, the *in vivo* efficacy of this combination is lacking. In the present study, we used a xenograft tumor model with human epithelial carcinoma A431 cells to test the effect of curcumin and visible light on tumor growth. It was found that tumor growth was significantly inhibited in mice that were i.p. injected with curcumin and consecutively irradiated with visible light. Furthermore, immunohistochemistry showed a reduction of Ki 67 expression, indicating a decrease of cycling cells and induction of apoptotic bodies. The effect on apoptosis was further confirmed by Western blot analysis showing enhanced activation of caspases-9. *Vice versa* inhibition of extracellular regulated kinases (ERK) 1/2 and epidermal growth factor receptor (EGF-R) was observed which may aid inhibition of proliferation and induction of apoptosis. In summary, the present findings suggest a combination of curcumin and light as a new therapeutic concept to increase the efficacy of curcumin in the treatment of cancer.

Key words: curcumin; UVA and visible light; A431 xenografts; tumor growth; apoptosis

Curcumin is the major constituent and pharmacologically active component of tumeric, a herbal powder which is used in traditionally Asian medicine. Turmeric and extracts for pharmacological use are derived from the ground rhizome of *Curcuma longa* (Zingiberaceae family). Curcumin has been shown to exhibit anti-inflammatory,[1,2] and anti-oxidative[1,3] properties. Furthermore, curcumin was found to inhibit cell proliferation and induce apoptosis demonstrated by the regulation of the Bcl proteins,[4,5] the release of cytochrome c[6,7] and the cleavage of procaspase-9[8] and -8[9,10] in a variety of cells *in vitro*. Moreover, curcumin is known to inhibit cell growth and survival associated kinases, such as PKB/Akt,[11,12] ERK1/2[12,13] and the EGF-receptor.[12,14] Thus, curcumin offers the potential to act as an anti-cancer drug. It has been shown that p.o. applied curcumin inhibits oral[15,16] esophageal,[17] forestomach,[18–20] duodenal,[19] and colon[19,21,22] carcinogenesis *in vivo*. Likewise, topical application inhibits skin tumor promotion.[18,23] However, p.o. administration of curcumin had no effect on mammary,[22,24] liver, kidney,[25] lung,[24,26] or prostate[27] tumors. These findings confirm pharmacokinetic studies showing a low absorption of curcumin from the gastrointestinal tract. It was shown that oral administration in rodents[28,29] and in humans[30] led to negligible amounts of curcumin in the plasma. Garcea *et al.*[31] found only traces of curcumin metabolites, but not curcumin itself, in human liver tissue after oral administration. Therefore, strategies that allow an increased systemic bioefficacy are favored. Earlier studies from our laboratory showed that curcumin at low concentrations inhibited proliferation and induced apoptosis in cultured skin cells only when combined with UVA or visible light.[32] In the present work, we have investigated whether this combined treatment inhibits tumor growth *in vivo*.

Material and methods

Cell culture and treatment

The human epidermoid carcinoma cell line A431 (American Type Culture Collection) was cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco, Karlsruhe, Germany) supplemented with 10% fetal calf serum (PAA, Coelbe, Germany) and 1% penicillin-streptomycin solution (Biochrom KG, Berlin, Germany) at 37°C in a 7.5% CO_2 atmosphere. For cell-treatment, 30 mg curcumin (Sigma, Deisenhofen, Germany) was dissolved in 1 ml DMSO. The stock solution was diluted in medium or PBS to concentrations ranging from 0.25 to 2 μg/ml. Cells were incubated for 1 hr with medium containing curcumin at 37°C in a 7.5% CO_2 atmosphere. Cells were irradiated with 1 J/cm^2 UVA (UV therapy system UV 3003 K, Waldmann, Villingen-Schwenningen, Germany) or 5 min with 5500 lx visible light (10 × 40 W lamps, distance 45 cm, emission spectrum: 400–550 nm; Philips GmbH, Hamburg, Germany). To avoid the formation of phototoxic products within the culture medium, PBS containing curcumin was used during irradiation. Nonirradiated controls were kept in the dark.

BrdU incorporation

To determine the replication rate, cells were cultured in microwell plates at a density of 2×10^4 cells per 0.33 cm^2. After 24 hr, cultures were treated with curcumin and light as mentioned earlier. Subsequently, 5-bromo-2′-deoxyuridine (BrdU) was added to the cells. The incorporation rate of BrdU was determined after 24 hr using a commercial ELISA test-kit (Roche, Mannheim, Germany). Briefly, cells were fixed and immune complexes were formed using peroxidase-coupled BrdU-antibodies. A colorimetric reaction with tetramethylbenzidine as a substrate gives rise of a reaction product measured at 450 nm using an ELISA reader (MR 5000, Dynatech, Guernsey, UK).

Cell viability

Cell viability was monitored by following the reduction of the dye AlamarBlue (Biosource, Karlsruhe, Germany) to the red fluorescing resofurin in response to cell metabolism. Therefore, cells were seeded in microwell plates (2×10^4 cells/0.33 cm^2) and treated with curcumin and light as mentioned earlier. The next day, AlamarBlue was added (10% v/v) to the cells and fluorescence was measured after 2 hr (excitation 530 nm, emission 620 nm) using a CytoFluor (series 4000, PerSeptive Biosystems, Framingham, MA).

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Apoptosis

Apoptosis was quantified on the basis of cytoplasmic histone-associated DNA fragments using the Cell Death Detection ELISA (Roche) according to the manufacturer's instructions. In brief, cells were cultured in microwell plates (2×10^4 cells/0.33 cm^2) and treated with curcumin and light as mentioned earlier. After 24 hr, the cytosolic fraction (200*g* supernatant) was used as antigen source in a sandwich enzyme-linked immunosorbent assay with primary anti-histone antibody coated to the microtiter plate and secondary anti-DNA antibody coupled to peroxidase. Optical density was measured at 530 nm in an ELISA reader (MR 5000, Dynatech).

Cytotoxicity

Membrane integrity was quantified using a cytotoxicity detection kit (Roche), which is based on the release of lactate dehydrogenase (LDH) from damaged cells. Briefly, A431 cells were cultivated in microwell plates (2×10^4 cells/0.33 cm^2) and treated with curcumin and light as aforementioned. The next day, cell-free supernatants were incubated with NAD^+, which is reduced by lactate dehydrogenase to $NADH/H^+$. Consecutively, $NADH/H^+$ reduces the yellow tetrazolium salt to a red-colored formazan salt. The amount of red color is proportional to the number of lyzed cells. For quantification, the absorbance of the reaction product was measured at 490 nm using an ELISA reader (MR 5000, Dynatech).

Animals and treatment

About 5×10^6 A431 cells were injected subcutaneously (s.c.) into the left and right flank of female Naval Medical Research Institute (NMRI) nude mice (5–6 weeks, 20–24 g; Harlan Winkelmann, Borchen, Germany). Mice were kept under specific pathogen-free conditions. Sterilized food and tap water were given ad libitum. Animals were anesthetized with ketamine/xylazine (100/10 mg/kg; i.p.: Pharmacia, Erlangen and BayerVital, Leverkusen, Germany) or isofluran (Abbott, Wiesbaden, Germany). For treatment, 5 mg curcumin was dissolved first in 50 µl ethanol and further diluted in 2 ml 1% methylcellulose (Sigma). Mice were injected i.p. twice a day with 200 µl of the suspension or methylcellulose solution. One group of curcumin and methylcellulose injected mice was additionally irradiated for 20 min with visible light (intensity as given earlier). The control groups were kept light-protected for 1 hr after injection. Tumor size was first measured after 10–12 days; afterward tumor volumes and body weights were determined twice a week. At the end of the experiment (25–29 days), anesthetized mice were sacrificed 0.5, 1, 4 or 24 hr after treatment. Tumors were excised and freed from necrotic tumor fluid. One part of the tumor was snap-frozen for Western blot analysis and the other part was placed in 4% paraformaldehyde for immunohistochemistry.

Experiments with human cancer xenograft mouse models were approved by the Regierungspräsidium Darmstadt (F49/34). All experiments were done in certified laboratories of the School of Medicine in Frankfurt/Main.

Immunohistochemical staining

For immunohistochemical staining, tumors were fixed in 4% paraformaldehyde, dehydrated, embedded in paraffin and sectioned using a Leica RM2125 rotary microtome (Leica Microsysthems, Wetzlar, Germany). Tumor sections were dried overnight at 37°C, deparaffinized in xylol, rehydrated in a series of graded alcohols, rinsed in destilled water followed by heat-induced epitope retrieval in 10 mM citrate buffer (DakoCytomation, Hamburg, Germany). The sections were then cooled for 30 min and washed 3 times with PBS.

Tumor proliferation was determined by Ki 67 staining, therefore, the appropriate antibody (clone MIB-1, 1:50 in PBS, DakoCytomation) was applied for 1 hr at room temperature. After 3 washes with PBS, the bound antibody was visualized with the

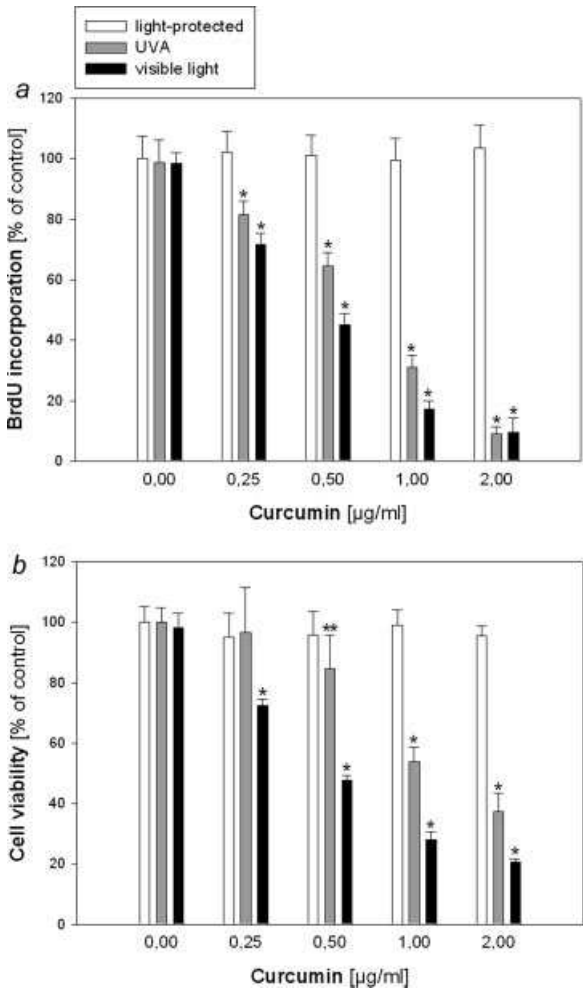

FIGURE 1 – Effect of curcumin and visible light or UVA on cell proliferation and viability. A431 cells were preincubated with curcumin and irradiated in the presence of curcumin as described in "Materials and methods" with 5500 lx visible light (5 min) (gray bars), 1 J/cm^2 UVA (black bars) or kept light-protected (white bars). (*a*) BrdU incorporation after 24 hr and (*b*) cell viability after 2 hr were determined. Control values were normalized to 100%. Each bar represents the mean of 8 independent experiments with standard deviation ($*p < 0.001$, $**p < 0.01$ *vs.* untreated control).

UltraTech HRP (AEC) Streptavidin-Biotin Detection System (Beckman Coulter, Marseille, France) according to the manufacturer's instructions. Finally, sections were counterstained using Mayer's hematoxylin (AppliChem, Darmstadt, Germany) and mounted with Aquatex (Merck, Darmstadt, Germany). Apoptotic cells were monitored by nuclei staining using bisbenzimide (1 µg/ml, Riedel de Häen, Seelze, Germany) for 10 min at room temperature. Consecutively, slides were washed and mounted with Aquatex.

Photographs were taken with a digital camera (Cyber-shot, Sony, Berlin, Germany) connected to an Axioscope (Zeiss, Oberkochen, Germany). Calculations were performed by counting positive cells per 700 (Ki 67) or 400 (bisbenzimide) nuclei. In the case of bisbenzimide staining, apoptotic cells were assessed on the basis of changes in nuclear morphology, such as chromatin condensation and fragmentation (apoptotic bodies).

Western blot analysis

Cleavage of procaspase-8 and -9 and phosphorylation of PKB/Akt, ERK1/2 and EGF-R were detected by Western blot analysis. Tumor tissue was homogenized (ultrathurax IKA, Staufen,

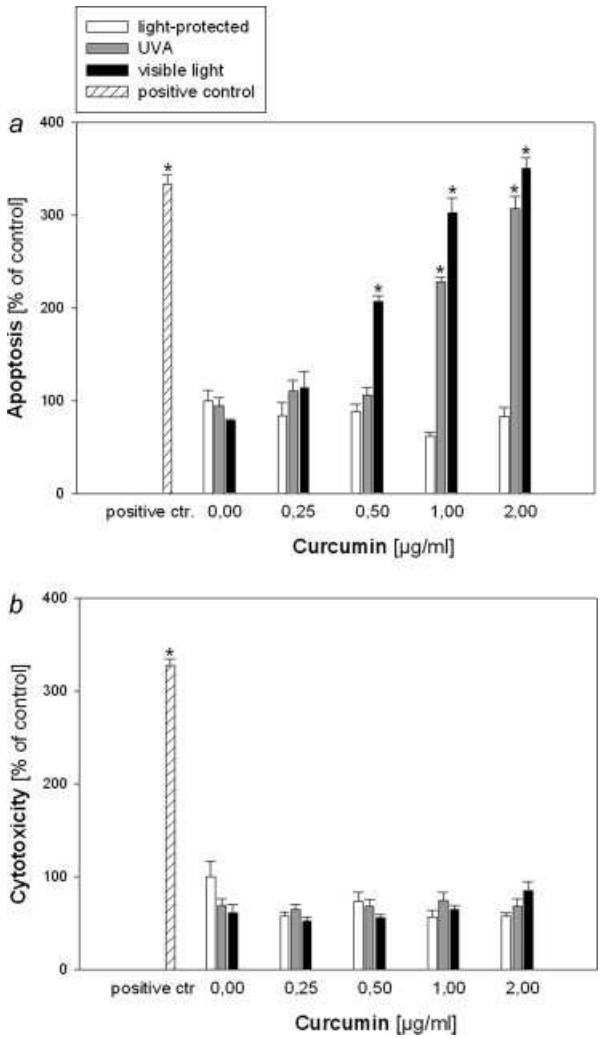

Figure 2 – Effect of curcumin and visible light or UVA on apoptosis induction and cytotoxity. A431 cells were preincubated with curcumin and irradiated in presence of curcumin as described in "Materials and methods" with 5500 lx visible light (5 min) (gray bars), 1 J/cm^2 UVA (black bars) or kept light-protected (white bars). (*a*) apoptosis and (*b*) cytotoxicity ELISA were carried out after 24 hr. A positive control for apoptosis was provided within the ELISA. Supernatants of cells treated with 1% Triton X 100 (gray bars) served as positive control for cytotoxity. Control values were normalized to 100%. Each bar represents the mean of 4 independent experiments with standard deviation (*$p < 0.005$ *vs.* untreated control).

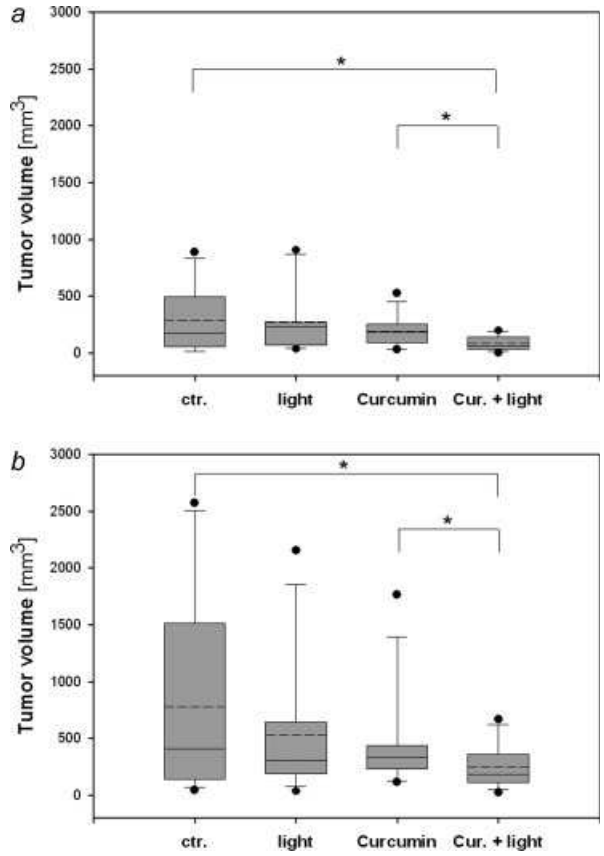

Figure 3 – Effect of curcumin and visible light on A431 tumor growth. Immunodeficient mice were injected s.c. with A431 cells and treated twice daily with curcumin (i.p.), light or curcumin (i.p.) and light. The tumor volumes are shown after (*a*) 12 and (*b*) 17 days. Twelve individual tumor volumes are shown in boxplots with 25% and 75% (boxes), 5% and 95% (bar errors) quantiles, the median (line within the box) as well as the mean (dashed line). Dots represent outside values (*$p < 0.05$ *vs.* control).

Germany) in cell lysis buffer (Cell Signaling Technology, Frankfurt, Germany). After centrifugation, protein concentrations of the supernatants were determined (DC Protein Assay, BioRad, Munich, Germany) and diluted with 3×SDS sample buffer (187.5 mM Tris-HCl [pH 6.8], 6% SDS, 30% glycerol, 150 mM DTT, 0.3% bromophenol blue) to obtain equal protein amounts (10 µg/15 µl). Samples were boiled for 5 min and separated on a 12% SDS polyacrylamide gel. For immunoblotting, proteins were transferred to a PVDF membrane (Millipore, Bedford, MA). The membrane was blocked in blocking buffer (Tris-buffered saline [pH 7.6], 0.1% Tween-20, and 5% bovine serum albumin or nonfat dry milk) for at least 1 hr, followed by overnight incubation with antibodies against activated EGF-R (Transduction Laboratories, Lexington, KY), β-actin (Sigma), cleaved caspase-8, -9, total and phospho-specific PKB/Akt or ERK1/2 (Cell Signaling Technology) in blocking buffer at 4°C. The bound primary antibodies were detected using IgG-horseradish peroxidase-conjugate (Cell Signaling Technology) and visualized with a chemiluminescence detection system (LumiGlo, Cell Signaling Technology). Western blots were quantified by densitometry using ImageJ 1.37v software (NIH, Bethesda, MD).

Statistics

The Mann-Whitney-*U*-test was used for statistical analysis. $p < 0.05$ was considered to be statistically significant. Data are given as means ± standard deviations.

Results

Curcumin combined with light inhibited cell proliferation and reduced cell viability in A431 cells

First, the effects of curcumin and light on A431 cells *in vitro* were examined. A431 cells were preincubated with curcumin (0.25–2 µg/ml) for 1 hr and then irradiated with UVA (1 J/cm^2) or visible light (5,500 lx, 5 min). Irradiation of curcumin treated cells with 1 J/cm^2 UVA or visible light gave rise to a clear inhibition of cell proliferation as measured by BrdU-incorporation (Fig. 1*a*). The rate of inhibition depended on the concentration of curcumin and was at first visible at 0.25 µg/ml. The dose of visible light showed stronger effects than 1 J/cm^2 UVA when applied with equal amounts of curcumin. Whereas 1 µg/ml curcumin in combination with visible light reduced proliferation to 17.3%, the reduction by the UVA combination was 31.1%. In light-protected but

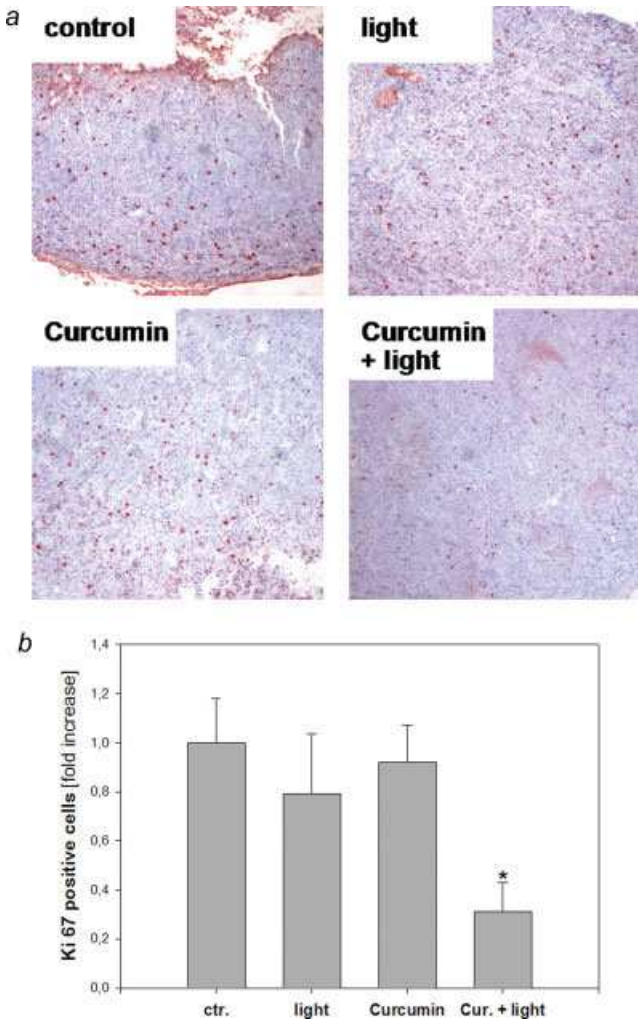

FIGURE 4 – Effect of curcumin and light on Ki 67 expression in A431 tumors. Immunodeficient mice bearing s.c. A431 xenografts were treated with curcumin (i.p.), light or curcumin (i.p.) and light. (*a*) Immunohistological staining for Ki 67 was performed with tumors obtained 24 hr after treatment. (*b*) Quantification of positive cells was assessed as described in "Material and methods." The control was normalized to 1. Each bar represents the mean of 5 sections with standard deviation (*$p < 0.005$ *vs.* control).

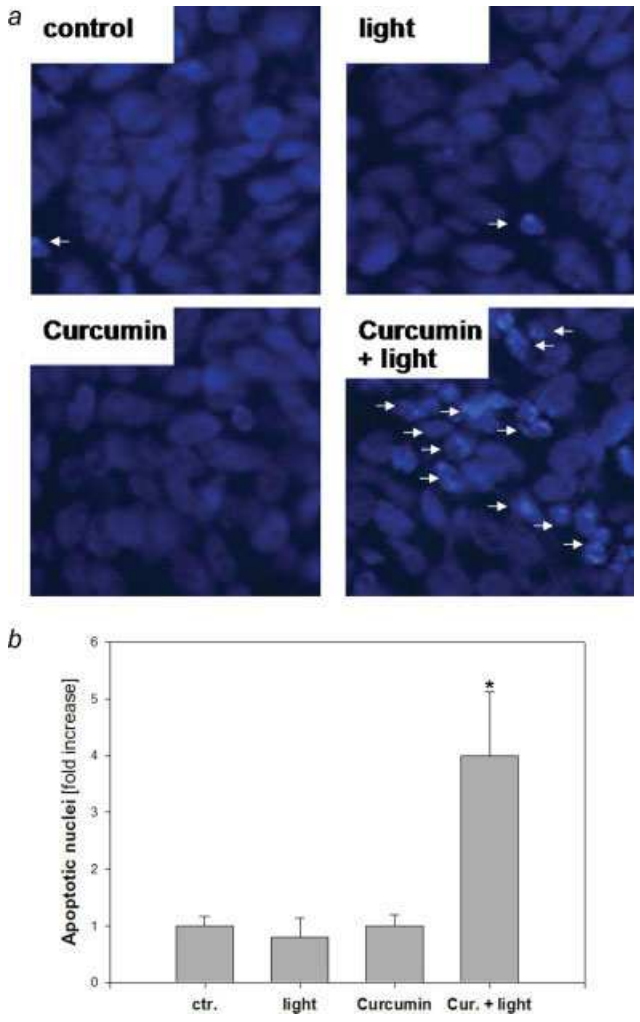

FIGURE 5 – Effect of curcumin and light on the induction of apoptotic nuclei in A431 tumors. Immunodeficient mice bearing s.c. A431 xenografts were treated with curcumin (i.p.), light or curcumin (i.p.) and light. (*a*) Bisbenzimide staining of nuclei was carried out with tumors obtained 24 hr after treatment. (*b*) Quantification of positive cells was assessed as described in "Material and methods." Apoptotic bodies are marked with arrows. The control was normalized to 1. Each bar represents the mean of 4 sections with standard deviation (*$p < 0.01$ *vs.* control). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

curcumin-treated cultures, no significant proliferation inhibiting effect was found in the concentration range tested. Corresponding results were obtained by measuring the cell viability after curcumin/light treatment using Alamar Blue™ (Fig. 1*b*). A reduction in metabolic rate was detected in curcumin/light-treated cultures but not in the absence of light. Likewise, the combination with visible light resulted in a stronger decrease in cell viability than with UVA, namely, 50% and 35%, respectively.

Curcumin combined with light induced apoptosis without cytotoxic effects

To investigate whether the observed proliferation inhibiting effect of curcumin in the presence of light is due to apoptosis induction or purely a result of toxic membrane damages we incubated A431 cells with different concentrations of curcumin (0.25–2 μg/ml) and irradiated the cells with UVA or visible light. After 24 hr, the rate of apoptosis was determined on the basis of histone-associated DNA fragments. Figure 2*a* shows that curcumin in combination with UVA or visible light induced apoptosis in a concentration dependent manner. Light-protected cells showed no apoptotic activity in response to curcumin up to 2 μg/ml. About 0.5 μg/ml curcumin gave rise to a massive apoptosis induction in combination with visible light, whereas UVA had no effect at this concentration. Higher curcumin concentrations induced apoptosis in combination with visible as well as UVA light. Cell membrane integrity, as assessed by the release of LDH into the cell culture supernatant, was not changed by curcumin alone and also not by a combination of light and curcumin up to 2 μg/ml indicating that the observed effects are not due to general cytotoxicity (Fig. 2*b*).

Curcumin combined with visible light inhibited the growth of A431 tumors in mice

NMRI nude mice were subcutaneously injected with A431 cells into the left and right flank. The treatment twice a day with methylcellulose (control), methylcellulose/visible light, curcumin or the combination of curcumin and visible light was started the same day. Tumor volume was measured after 12 (Fig. 3*a*) and 17 days (Fig. 3*b*). Data presented show that only curcumin/light treat-

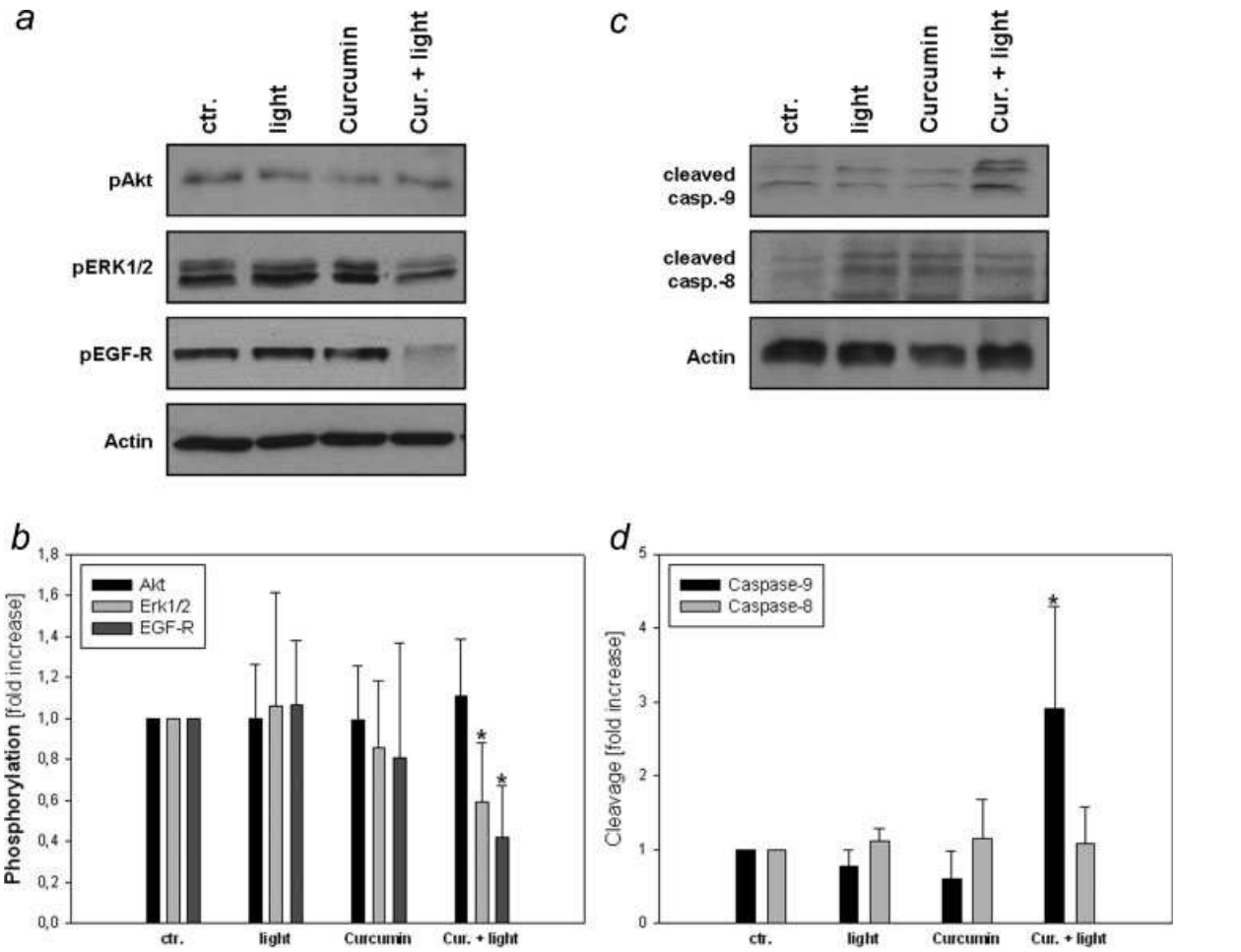

Figure 6 – Effect of curcumin and light on ERK1/2, PKB/Akt EGF-R, caspase-9 and -8 in A431 tumors. Immunodeficient mice bearing s.c. A431 xenografts were treated with curcumin (i.p.), light or curcumin (i.p.) and light. Western blot analyses were performed (*a*) against p (phospho) Akt (Ser; 60 kDa), pERK1/2 (42 kDa), pEGF-R (175 kDa) after 1 hr and (*c*) against cleaved caspase (casp.) -9 (35/37 kDa) and -8 (41/43 kDa) after 4 hr. β-actin served as loading control. (*b, d*) Evaluation was performed as described in "Material and methods." The control was normalized to 1. Each bar represents the mean of 4 values with standard deviation (*$p < 0.01$ *vs.* control).

ment evoked a significant tumor growth inhibition compared to the control group. The average tumor volume at Day 12 in curcumin/light treated mice was reduced by approximately 70% in comparison to the control group, namely, 83 ± 7 mm^3 and 285 ± 28 mm^3, respectively. The tumor volume reduction of the curcumin-injected but nonirradiated group was not significant ($p = 0.16$ versus control), whereas the curcumin/light group also showed a significant difference versus this group ($p = 0.05$). The relative ratio between tumor volumes of the different groups was similar after 17 days. There was no effect of the various treatments on body weight (data not shown).

Curcumin combined with visible light inhibited the Ki 67 expression and induced apoptotic nuclei in A431 tumors of immunodeficient mice

To determine the proliferation rate in tumors, immunohistological sections were stained for Ki 67, which is expressed in all phases of the cell cycle except G0. Tumors isolated 24 hr after treatment showed a significant decrease in Ki 67 positive cells when treated with curcumin and light (Figs. 4*a* and 4*b*). Corresponding to the data on tumor growth, curcumin/light treatment inhibited proliferation by about 70%, whereas light and curcumin alone had no effect on Ki 67 expression.

Furthermore, we tested whether a combination of light and curcumin triggers apoptosis. Thus, tumors were stained with bisbenzimide to observe the formation of apoptotic bodies. We found a significant 4-fold increase of apoptotic nuclei in tumor tissue of curcumin-injected and irradiated mice after 24 hr (Figs. 5*a* and 5*b*). Curcumin alone and irradiation without curcumin treatment did not show significant effects on the formation of apoptotic bodies.

Curcumin combined with visible light inhibited ERK1/2 and EGF receptor and activated caspase-9 in A431 tumors

To study the impact of curcumin and light on targets involved in apoptosis and proliferation, tumors were isolated 0.5, 1 and 4 hr after treatment. These samples were subjected to Western blotting for phosphorylated Akt/PKB, ERK1/2 and EGF receptor and cleaved procaspase-8 and -9. Whereas samples obtained after 30 min did not show any differences (data not shown), samples isolated after 1 hr revealed a significant decrease in phosphorylation of ERK1/2 and EGF-R (but not PKB/Akt) only in the curcumin/light group (Figs. 6*a* and 6*b*). Activation of caspase-9 was first detectable after 4 hr (data after 0.5, 1 hr not shown) in the tumors from the curcumin/light group, whereas caspase-8 did not show any differences within the time range tested (Figs. 6*c* and 6*d*). Treatment with curcumin and irradiation alone did not reveal significant changes in the activation of the aforementioned kinases and caspases.

Discussion

It is well known that curcumin exhibits growth inhibiting and apoptosis inducing properties in different cells suggesting curcumin to be a potent anti-cancer agent. However, the therapeutic use of curcumin is hampered by its poor gastrointestinal resorption. After oral administration of 400 mg curcumin to rats, less than 5 μg/ml were detected in blood, and 20 μg/tissue in liver and kidney.[28] Similarly, a test with human volunteers showed almost no compound traces within the serum after intake of 2 g curcumin.[30] *In vitro* studies demonstrated that concentrations of 10–150 μM (3.7–55 μg/ml) are necessary for pharmacological effects.[5,33] Therefore, options to enhance the bioavailability of curcumin are desirable. In this context, earlier studies from our laboratory showed that in HaCaT keratinocytes, curcumin at low concentrations (0.2–1 μg/ml) offered proliferation inhibition and apoptosis induction only in combination with UVA or visible light.[32] The aim of the present study was to determine whether the combination of curcumin and light exhibits similarly strong effects *in vivo*. A nude mouse xenograft animal model was used allowing the irradiation through the skin of s.c. injected A-431 xenografts. From the *in vitro* tests, it was found that curcumin at 0.2 and 2 μg/ml in combination with UVA or visible light caused significant proliferation inhibition, reduced cell viability and induced apoptosis without membrane damage in A431 cells. Subsequent *in vivo* studies prove that treatment twice a day with curcumin (500 μg/200 μl; i.p.) leads to a retarded tumor growth only when combined with visible light irradiation. Accordingly, tumors of the curcumin/light group also showed decreased Ki 67 expression and additional apoptotic nuclei.

Furthermore, we investigated the activation status of PKB/Akt, a key molecule of cell survival and the proliferation associated MAP-kinase ERK1/2 *in vivo*, which become inhibited *in vitro* by low concentrations of curcumin in combination with light[32] and by curcumin alone at high concentrations.[11,12] In our *in vivo* model, we showed that curcumin/light leads to a significant decrease of ERK1/2 activation 1 hr after treatment, but no effect on PKB/Akt phosphorylation within 4 hr. Chun *et al.*[34] also demonstrated suppression of ERK1/2 *in vivo* namely in mouse skin after topical application of curcumin. Kinases are activated by, for example PTK-receptors such as the EGF receptor which is inhibited by curcumin[12,14] or curcumin and light[32] *in vitro*. In our *in vivo* model, we showed that EGF-R is likewise dephosphorylated 1 hr after curcumin/light treatment.

Furthermore, most of the *in vitro* studies demonstrate the onset of the intrinsic pathway in curcumin-mediated apoptosis by showing, for example, the release of cytochrome c[6,7] and the regulation of proteins from the bcl-2 family.[4,11] Our findings confirm the onset of intrinsic apoptosis by showing that curcumin/light in tumors of xenograft mice induces the activation of the intrinsic initiator caspase-9, but not caspase-8 within 4 hr. Similarly, our recent studies showed activation of caspase-9 followed by delayed caspase-8 activation in HaCaT keratinocytes.[32] It is well known that EGF-R inhibition leads to induction of intrinsic apoptosis through Bad activation[35] suggesting that curcumin/light acts in this manner.

In summary, we have demonstrated light-induced sensitization of curcumin *in vivo*. Our study shows that a combination of light and curcumin amplifies the anti-growth and proapoptotic effects of curcumin in a tumor model. This observation suggests a novel therapeutic approach in photodynamic tumor therapy.

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