Photodynamic Therapy Potentiates the Effects of Curcumin on Pediatric Epithelial Liver Tumor Cells

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Abstract. *Background/Aim: Curcumin (CUM) is a promising agent in complementary oncology. The present study analyzed the photoactive properties of curcumin on pediatric epithelial liver tumor cell lines. Materials and Methods: Hepatoblastoma cell lines (HuH6, HepT1) and hepatocellular carcinoma cell lines (HepG2, HC-AFW1) were treated with curcumin and exposed to blue light (phototherapy, 480 nm, 300 W). Cell viability (MTT tests), cellular oxidative stress (production of reactive oxygen species (ROS)) and cellular uptake/degradation of curcumin were analyzed. Results: Significant loss of viability resulted from 24-48 h incubation with curcumin. With photodynamic therapy (PDT), even short time incubation (1 h) with curcumin resulted in significantly lower half maximal inhibitory concentration (IC_{50}) ($p<0.001$, two-way ANOVA). Significant ROS production was observed with PDT and curcumin. Conclusion: Phototherapy strongly enhances the anticancer properties of curcumin in pediatric solid liver tumors in vitro.*

Human hepatoblastoma (HB) is the most common primary malignant liver tumor in infants and children (1). Despite progresses in long-term survival, advanced HB, relapsed or metastasized tumors still are associated with a poor prognosis with an overall survival rate of 53% and a disease-free survival of 36% (2, 3). Hepatocellular carcinoma (HCC) is a rare tumor entity in children. The outcome of these patients is even worse compared to Hepatoblastoma (4). The 5-year overall survival is 28% and the event-free survival >75 months is only 17%.

Preoperative chemotherapy in HB, as well as in pediatric hepatocellular carcinoma (pHCC), should ideally lead to significant reduction of tumor burden to enable surgical resectability. In addition, the aim of chemotherapy is to eliminate free circulating tumor cells even in intraoperative situations, in order to prevent tumor cell dissemination. However, advanced or relapsed tumors still represent an unsolved problem due to the phenomena of drug resistance (5, 6).

Curcumin ((CUM) 1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione), also known as diferuloymethane, is a yellow-orange dye derived from the rhizome of the plant *Curcuma longa*. It is one of the most extensively studied phytochemicals in complementary oncology. In tumors, it induces apoptosis, inhibits cell proliferation and angiogenesis (7). Furthermore, it efficiently affects several pathways associated with cancer stem cell self-renewal like Notch, Wnt, Hedgehog, STAT and interleukin' signaling (8). Furthermore, it facilitates absorption of radiation between 350-500 nm and causes oxygen-dependent phototoxicity (9-11). We recently showed that curcumin inhibits the pediatric hepatocellular carcinoma cell line HC-AFW1 *in vitro* and, in combination with cisplatin, reduces the tumor growth of xenotransplantated HC-AFW1 tumors *in vivo* (12).

In this study, we analyzed the photodynamic properties of curcumin on hepatoblastoma and hepatocellular carcinoma cell lines *in vitro*, namely HuH6, HepT1, HepG2 and HC-AWF1 cells.

Materials and Methods

Drugs and phytochemicals. The native curcumin powder (CUR) (Jupiter Leys, Cochin, Kerala State, India) used in all formulations contained 82% curcumin, 16% demethoxycurcumin (DMC) and 2% bis-demethoxycurcumin (BDMC). Curcumin micelles (mic-CUR) were composed of 7% curcumin powder (equivalent to 6% curcumin) and 93% Tween-80 (Kolb, Hedingen, Switzerland) and were manufactured by AQUANOVA AG (Darmstadt, Germany). All percentages refer to weight.

Abbreviations: CUR, Curcumin; BDMC, bis- demethoxycurcumin; DMC, demethoxycurcumin; HB, hepatoblastoma; HCC, hepatocellular carcinoma; mic-CUR, micellar curcumin; PDT, photodynamic therapy; pHCC, pediatric hepatocellular carcinoma.

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Cell cultures. The following hepatoma cell lines were used: the HB cell lines HuH6 (mixed HB (13)) and HepT1 (multifocal embryonal HB (14)), the human pHCC cell lines HC-AFW1 (15) and HepG2 (LGC Promochen, HB8065, Salisbury, UK). The latter was initially reported as pediatric hepatocellular carcinoma of trabecular type; later, the authors corrected their report and claimed that HepG2 was derived from a HB (16, 17). Cells were cultured in DMEM (GIBCO BRL, Carlsbad, CA, USA) supplemented with 10% FCS and maintained in a humidified atmosphere containing 5% CO_2 at 37˚C. For sub-culturing, cells were detached from the culture surface using 0.05 % Trypsin-EDTA (Gibco® Life Technologies, Carlsbad, California, USA) or accutase (HC-AFW1 in Dulbecco's PBS containing 0.5 mM EDTA (PAA Laboratories GmbH, Cölbe, Germany) at 37˚C.

Viability assay. Viability tests were performed using MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromid) assays (AppliChem GmbH, Darmstadt, Germany). Cell cultures were disseminated in colorless culture media in 96-well plates in a concentration of 5×10^3 per well. For high-density culture, cells were concentrated five-fold. Due to their slower growth, HepT1 cell numbers were doubled in all experiments. After 48 h, CUR was added to the cells at increasing concentrations. The half maximal inhibitory concentrations (IC_{50}) were determined for long (48 h) and short (1 h) incubations with CUR. In photodynamic treatment (PDT) experiments, cells were exposed to blue light after 1, 3 or 6 h of incubation with CUR for 10 s with 480 nm blue light (λ=390-440 nm, 300 W xenon short-arc lamp; Karl Storz GmbH&Co., Tuttlingen, Germany).

Further analyses compared cell viability after CUR without PDT, PDT of the cells before treatment with CUR, PDT of the native CUR solution before added to the cells and PDT after 1.5 h CUR incubation.

All assays were performed 3 times in triplicates. Percentages of viability were calculated through normalization between background of cultures without cells and untreated cultures as control experiments. Dose-dependent viability curves were computed by sigmoidal curves with variable slope to determine IC_{50} using GraphPad Prism 4.00 (GraphPad Software, San Diego, CA, USA).

Detection of ROS and cell membrane integrity. Cells were cultured for 24 h and treated with increasing concentrations of CUR. Cells were exposed to blue light (480 nm) either directly or after washing out of CUR and incubated for 1 h. In a third experiment, CUR was first exposed to light and then added to cell culture for 1.5 h. After re-suspending the cells in colorless medium, measurements were performed with the BD FACSCalibur (BD FACSTM 7-Color Setup Beads; BD bioscience, San Jose, CA, USA).

Oxidative stress was measured by detection of ROS. ROS were detected with the cell-permeable, peroxide-sensitive fluorophore CellROX Orange (lambda Ex/Em=545/565 nm; Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. The dye is non-fluorescent when in a reduced state and exhibits bright orange fluorescence upon oxidation by ROS. The dye was added 24 h after PDT (5 μM per well). After 30 min incubation in the dark, cells were washed in PBS. Measurements were performed with BD FACSCalibur in the Fl3- and FL4-channel (lambda Ex/Em=520/572 nm for CellRox Orange).

For assessing cell viability by flow cytometry, 3 h after PDT of 7- Aminoactinomycin D (7-AAD, Viability Staining Solution; eBioscience Inc., San Diego, CA, USA) was used (5μl/well). 7-AAD enters the dead cell after loss of cell membrane integrity and, thus, acts as a marker for cell death. Due to overlapping emission spectra of CUR and 7-AAD, measurements were performed in the BD FACSCalibur in the FL3-channel with compensation.

Data files of flow cytometry were analyzed using FCS Express V3 Flow Cytometry software (De Novo Software, Los Angeles, CA, USA and BD FACSDiva (BD Biosciences) and GraphPad Prism (GraphPad Software, San Diego, CA, USA). Dead cells were gated out in the FSC/SSC dot plot. Histograms were normalized to the untreated control.

The uptake and degradation of curcumin was analyzed by measurements of curcumin fluorescence (lambda Ex/Em= 488/546 nm) in hepatoma cells with the BD FACSCalibur. Measurements of curcumin fluorescence in cells were measured after exposure to different CUR or mic-CUR concentrations (1 hour, 0, 1, 5, 10, 20 μg/ml) or directly after different incubation periods (1 or 3 hours, 5 μg/ml), respectively, 3 or 21 h after 3-h exposure to curcumin or mic-curcumin.

Fluorescent detection of CUR. Subconfluent and high-density cell cultures were treated with different concentrations of CUR (0, 1, 5, 10 μg/ml) in phenol-free DMEM (Gibco®) and were exposed to blue light for 10 s. Furthermore, different curcumin concentrations without cells were exposed to blue light and then added to the cell cultures. The fluorescence of curcumin was measured for 0.5 s (lambda Ex/Em=485/535, Luminometer Victor™ 1420 multilabel counter; Wallace, Freiburg, Germany) directly, 24 h hours or 48 hours after treatment in cell-fee medium with (1 and 10 s) and without blue light irradiation.

Statistical analyses. Data analysis was carried out using GraphPad Prism 4.00. In MTT, IC_{50} was calculated from the sigmoid dose response curves with variable slopes. In fluorescence measurements, LOG half maximal effective concentration (EC_{50})-values were calculated on the basis of sigmoidal dose-response curves with variable slopes. The obtained curves on hepatoma cells for each treatment were compared with respect to their IC_{50} or $LOGEC_{50}$-values and slope and the *p*-value was determined with 95% confidence intervals (CI). Comparison of two regression curves was performed by the F-test and a significant difference was obtained at *p*-values <0.05. All numeric data are expressed as means and standard deviations (SD).

Results

Curcumin in subconfluent and in high-density cell cultures. After 48 h of incubation with native CUR and mic-CUR, respectively, viability in all tested cells decreased in a concentration-dependent manner (Figure 1) in subconfluent, as well as in high-density cultures. Mic-CUR normally is used to enhance the extremely low bioavailability of native curcumin after oral application. CUR is released from the shattering micelles and enters the bloodstream. Herein, we showed that curcumin release from micelles directly to the tumor cells is also possible. The difference between the IC_{50} of curcumin and mic-curcumin in subconfluent cultures did not reach statistical significance (Table I).

Additive effects of short-time curcumin in combination with PDT on cell viability. In vivo, the metabolic half-life of CUR after oral application is much shorter than 48 h; therefore, we analyzed the effects of short curcumin incubation. After 1.5 h of incubation

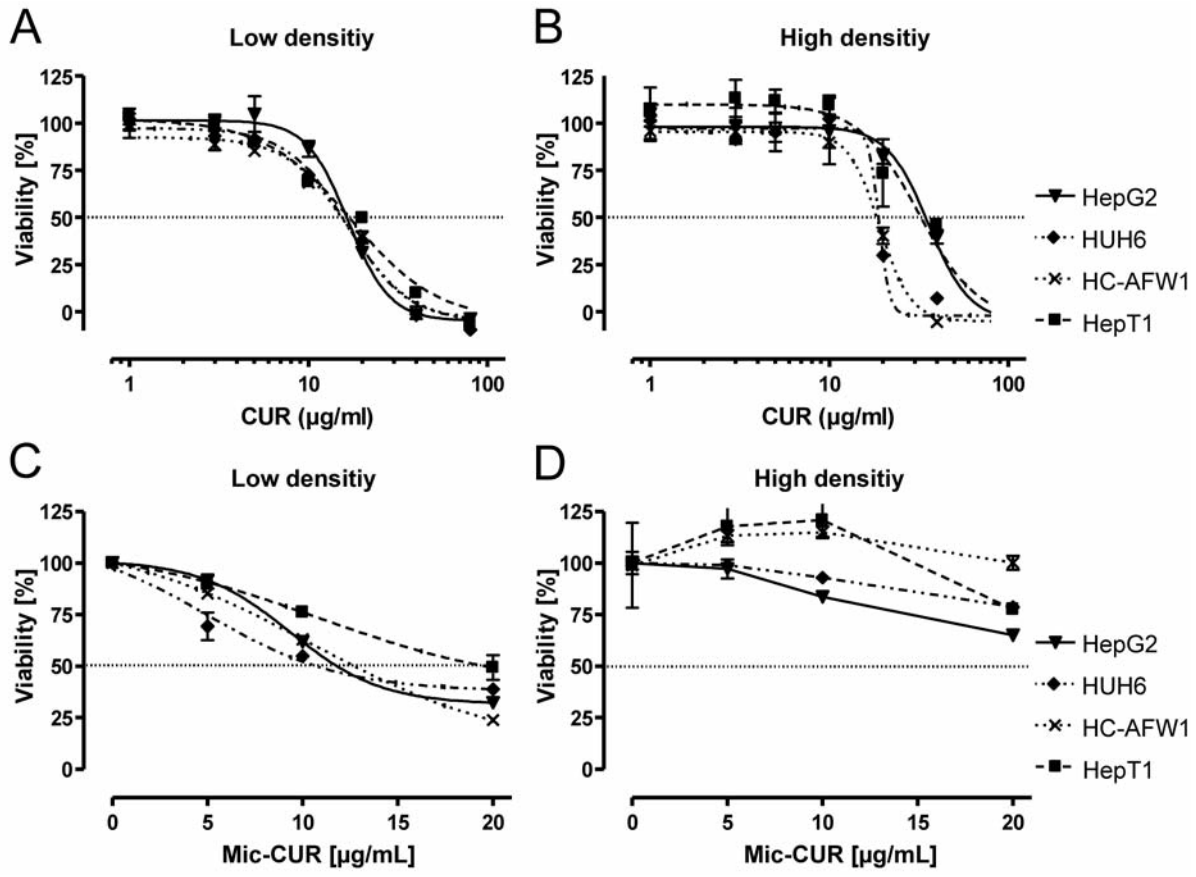

Figure 1. *Effects of native (A, B) and micellar curcumin (C, D) on low/ (LD) (A,C) and high/density (HD) (B, D) culture of HB and HCC cells.*

with native CUR and mic-CUR, respectively, the viability decreased in HC-AFW1 cells and HepT1 cells only minimally (Figure 2 A-D). Within the tested curcumin concentration, no IC_{50} could be reached at the short incubation period. Thus, longer exposure times of tumor cells to curcumin *in vivo* have to be achieved *via* high frequency-adjusted curcumin. Another possibility to enhance the CUR effects is PDT.

As previously reported, CUR acts as a photosensitizer, *e.g.* in murine leukemic cells (9). In combination with exposure to blue light (10 sec), the effect on cell viability (IC_{50}) is enhanced many times over (Figure 2A-D). For all tested cell lines, the decrease of viability due to short-time CUR and PDT was significant, compared to CUR without PDT (Table II).

Additionally to MTT tests, flow cytometry viability tests were performed with 7-AAD staining to rule out the rate of dead cells after curcumin treatment with or without PDT (Figure 2E-H). Compared to solitary curcumin incubation for 1.5 h in increasing concentrations, additional PDT led to significantly higher amounts of 7-AAD-positive cells (Figure 2E-H).

To analyze the impact of duration of CUR incubation before PDT, IC_{50} were compared in cultures after 1, 3 and 6 h of pre-incubation of the cells with CUR (Table III). As shown in Figure 3, an early PDT resulted in significant steep slopes of curves in all cell lines (left-shifted curves to lower concentrations). The higher loss of viability after shorter curcumin incubation indicates a different mechanism of action than in the curcumin incubation without PDT.

Curcumin is degraded by exposure to visible light, which results in a reduced efficacy (18). In our experiments, after extracellular exposure to blue light, curcumin is no longer able to significantly decrease cell viability during short incubation in combination with PDT (Figure 4). However, the photodegradation proceeds very fast with singlet oxygen formation, which may promote cell death (19).

To rule out possible mechanisms of viability reduction after curcumin and PDT, the development of ROS was measured. Without blue light exposure, in HC-AFW1 and HepT1 cells there was no increase of ROS, while in HepG2 and HuH6 cells there was very mild increase of ROS in the presence of CUR. After PDT, in all treated cell lines, the amount of ROS-positive cells increased to 73.4-99.6% (Figure 5).

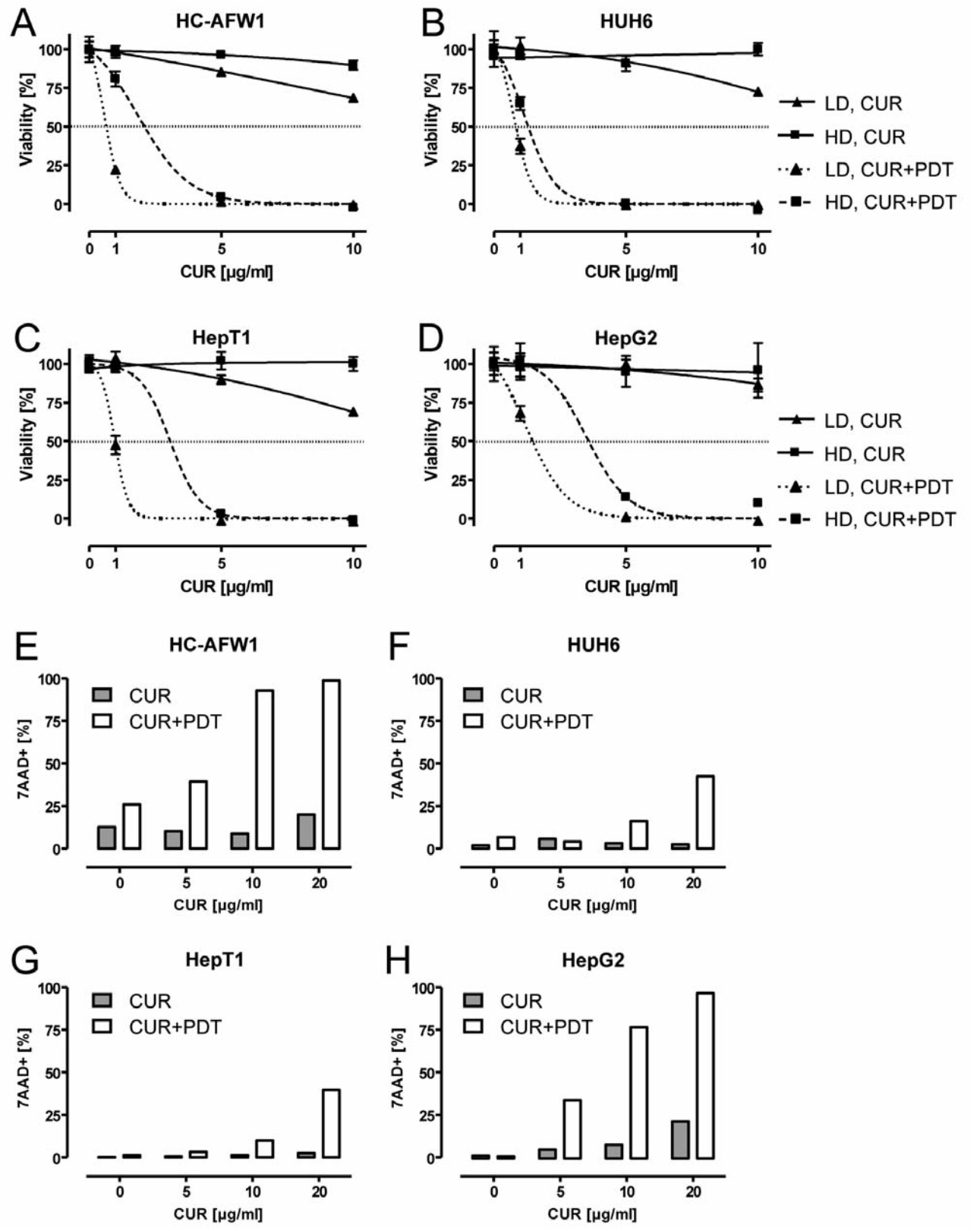

Figure 2. *PDT in combination with short-time incubation of native CUR leads to significant viability loss in subclonfluent in high-density cell cultures (A-D). After PDT CUR, the amount of 7AAD-positive cells is significantly higher after PDT + compared to CUR without PDT (E-H).*

Table I. *Reduction of viability by CUR.*

	HC-AFW1 IC_{50} (95%CI)	HuH6 IC_{50} (95%CI)	HepT1 IC_{50} (95%CI)	HepG2 IC_{50} (95%CI)
CUR, LD	16.95 (14.19-20.25)	16.43 (14.45-18.69)	17.37 (13.74-21.96)	16.64 (15.39-17.98)
CUR, HD	19.17 (18.25-20.13)	19.03 (18.56-22.96)	31.50 (22.48-44.15)	35.86 (29.76-43.21)
Mic-CUR, LD	10.7 (8.07-11.92)	11.8 (9.67-13.55)	12.7 (11.22-13.04)	19.5 (17.91-21.53)

IC_{50}, Half maximal inhibitory concentration (μg/ml); 95%CI, confidence interval; CUR, curcumin; LD, low-density cell cultures; HD, high-density cell cultures; mic-CUR, micellar curcumin.

Table II. *PDT raises the effects of CUR on cell viability.*

	HC-AFW1 IC_{50} (95%CI)	HuH6 IC_{50} (95%CI)	HepT1 IC_{50} (95%CI)	HepG2 IC_{50} (95%CI)
LD	16.95 (14.19-20.25)	16.34 (14.45-18.69)	17.37 (13.74-21.96)	16.64 (15.39-17.98)
HD	19.16 (17.34-22.9)	17.77 (12.34-24.56)	31.50 (22.48-4.15)	35.86 (29.76-43.21
LD, PDT	0.65 (0.27-0.85)*	0.78 (0.55-1.08)*	0.97 (0.56-1.12)*	1.14 (0.96-1.52)*
HD, PDT	2.10 (1.73-2.38)*	1.30 (0.93-1.86)*	3.04 (2.86-3.59)*	3.58 (3.22-4.01)*

IC_{50}, Half maximal inhibitory concentration (μg/ml); 95%CI, confidence interval; LD, low-density cell cultures; HD, high-density cell cultures; PDT, photodynamic therapy. *IC_{50}, two-way ANOVA, $p<0.0001$.

Table III. *The influence of exposure time to CUR/PDT combination.*

Time	HC-AFW1 IC_{50} (95%CI)	HuH6 IC_{50} (95%CI)	HepT1 IC_{50} (95%CI)	HepG2 IC_{50} (95%CI)
1 h	2.93 (2.87-3.15)	2.20 (2.06-2.54)	2.89 (2.35-3.97)	3.70 (3.52-3.87)
3 h	4.75 (4.21-5.02)	3.78 (3.19-4.64)	4.09 (3.68-4.64)	4.58 (4.3.94-4.83)
6 h	5.50 (5.01-5.65)	4.36 (3.96-4.49)	6.03 (5.50-6.82)	6.04 (5.87-6.27)

IC_{50}, Half maximal inhibitory concentration (μg/ml); 95%CI, confidence interval; LD, low-density cell cultures; HD, high-density cell cultures; regression analysis, F-test, $p<0.05$.

In further experiments, curcumin uptake in cells and degradation was analyzed. To this end, curcumin fluorescence was measured in flow cytometry after 1 h incubation with increasing curcumin concentrations. The respective mean fluorescence index (MFI) was higher with exposure to higher concentrations of CUR. The intracellular uptake of micellar curcumin seems, somehow, to be limited as there was no increase of MFI beyond 10 μg/ml; the highest uptake of CUR occurs in the first hour of exposure and decreases fast during the first 3 h (Figure 6).

Furthermore, we could express the metabolisation of curcumin by subconfluent and high-density hepatoma cultures by means of extracellular curcumin fluorescence detection, thus showing that more curcumin is metabolized by high-density cultures but without any significant increase of metabolisation after 48 h, compared to 24 h (Figure 6).

In addition, we showed that the fluorescence intensity of curcumin is rapidly and significantly reduced even by very short blue-light exposure ($p<0.0001$) (Figure 7).

Discussion

To overcome the poor prognoses of high-risk epithelial liver tumors, research for new treatment strategies or amendments to standard chemotherapy schemes with few side-effects, synergism to chemotherapeutic agents and a wide range of action is required. Curcumin is one of the most promising agents, having chemoprotective properties and multiple targets in tumor cells (20-22). In previous studies, we demonstrated its anti-tumoral properties like inhibition of NF-κB and beta-catenin in an *in vivo* model of a human pediatric hepatocellular carcinoma in combination with cisplatin (23). The low enteral bioavailability of native curcumin (24) was overcome by diverse changes in its galenics, such as binding to nanoscale polymer carriers or in combination with piperine (25). Highest curcumin concentrations were measured after oral application of micellar curcumin (23, 26). A less investigated possible anti-tumoral mechanism is caused by the fluorescence of curcumin: as a phenolic pigment, it is used as

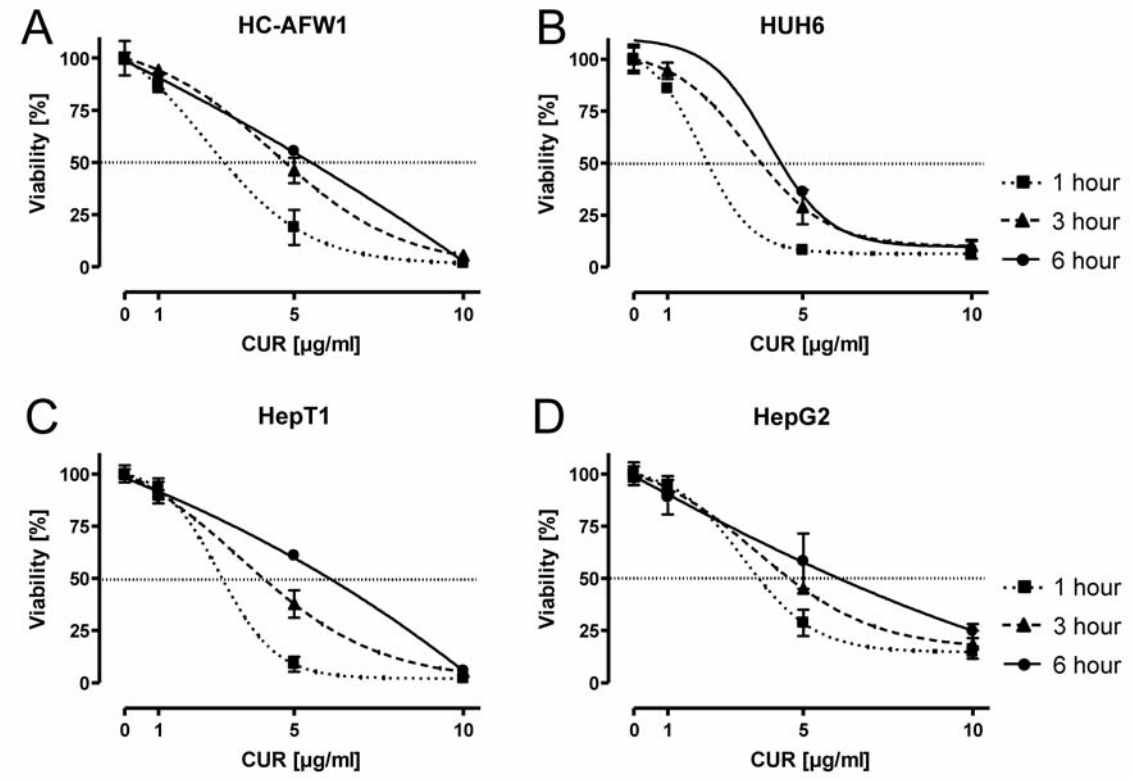

Figure 3. *Hepatoma cells received PDT after 1, 3 or 6 h CUR incubation. Short CUR incubation led to highest loss of viability.*

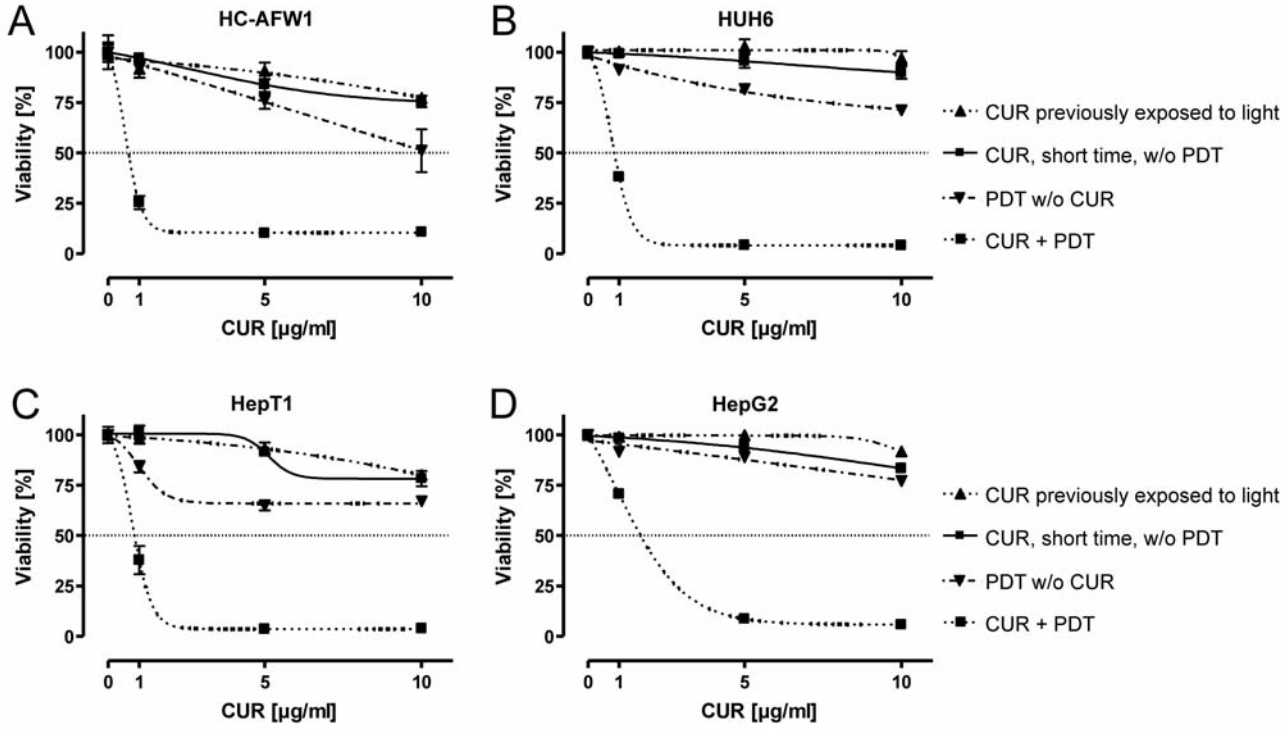

Figure 4. *Only combination of short-time CUR together with PDT led to loss of viabilty; short-time CUR alone, PDT without CUR or light-exposed CUR did not cause any significant loss of viability to the cells.*

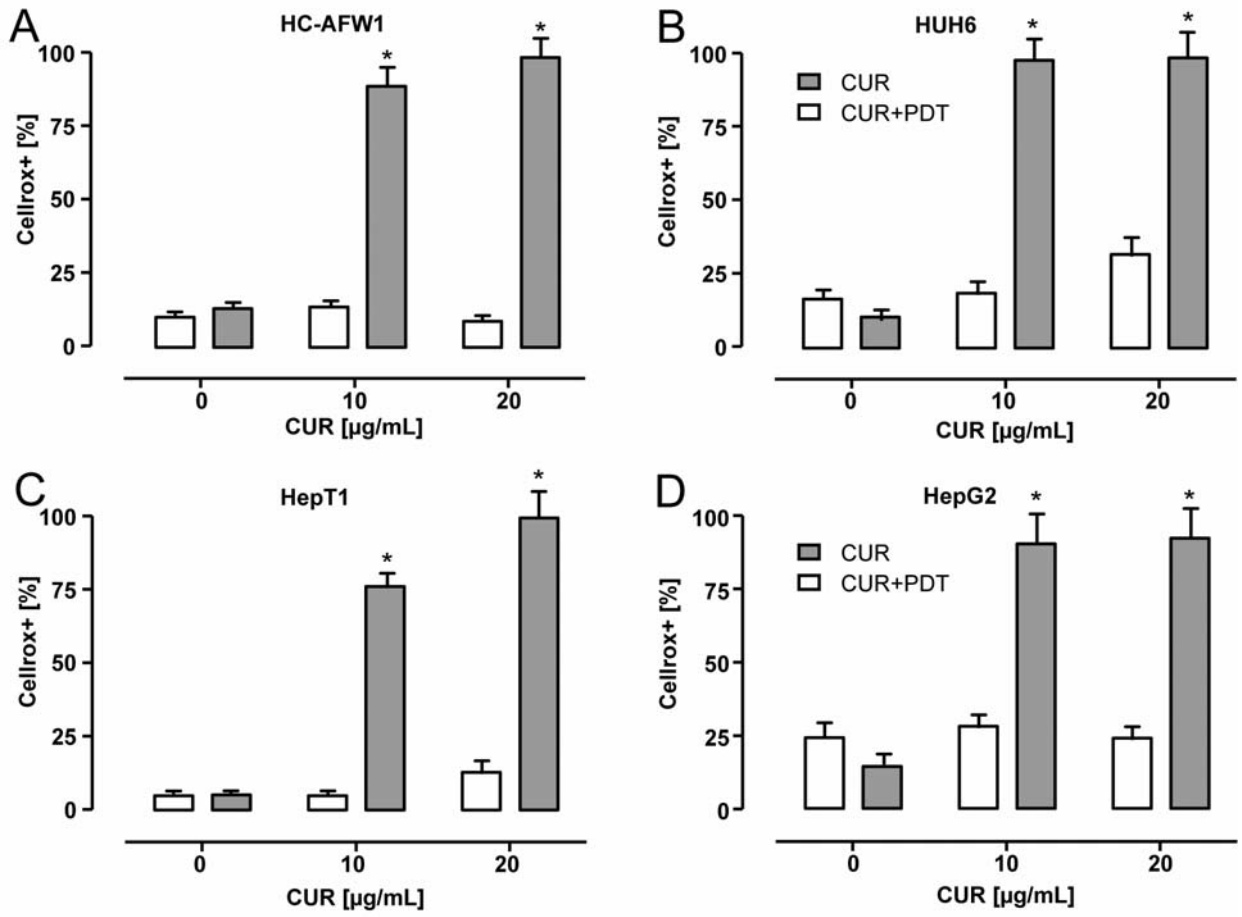

Figure 5. *CUR in combination with PDT leads to significantly higher ROS production than without PDT in all cell lines. *p<0.05.*

a yellow-orange dye that exhibits a variety of photochemical activity, including photoxicity to various cells. In 1994, it was proven that curcumin can lead to production of ROS in bacteria or mammalian cells upon irradiation with visible light >400 nm (9). *In vitro*, its cytotoxicity against several tumor cells could be strongly enhanced by irradiation with visible or blue light (27). This was proven especially for nasopharyngeal carcinoma cells (10), skin fibroblasts, melanocytes, melanoma cells (28), HeLa cells (29) and lung carcinoma cells. The cytotoxic effects of low doses of curcumin are amplified with irradiation with light of wavelengths near the absorbance maximum of curcumin (27). While in a neutral solution, the absorption band has a peak at about 420 nm; in basic solutions the band peak is about 480-500 nm. Similar to other works with other cell lines, we showed that the combination with blue light irradiation (480 nm) amplifies the cytotoxicity of curcumin to all tested epithelial liver tumor cells. Not only the needed IC_{50} were reduced drastically but, also, the needed interaction time of cells with curcumin could be shortened significantly. Best effects were seen after light exposure early after curcumin treatment suggesting a fast degradation of cellularly incorporated curcumin. Most publications on curcumin therapy in combination with PDT (UVA, blue light) deal with its antimicrobial or anti-tumoral activity on mucosal or skin lesions or on biofilms or on tumor cell cultures (30-33). Only one study described the effects of curcumin with phototherapy (visible light) in a murine model of a human subcutaneous tumor (28). This is stunning because the influence of low curcumin concentrations combined with PDT may compensate the low bioavailability and fast degradation of curcumin *in vivo*. *In vivo*, the relevant serum and organ concentrations of curcumin decrease rapidly during the first 5 h after oral application of micellar curcumin (23). The herein reported *in vitro* analyses of fast intracellular curcumin uptake during the first hour and fast degradation within 3 hfits to these findings. Furthermore, our studies revealed a fast metabolisation of curcumin in the cells. By means of subsequent PDT, shortly after administration of curcumin, this limiting effect might be cancelled out. Transferring these results in an *in vivo* model, after tumor resection, the

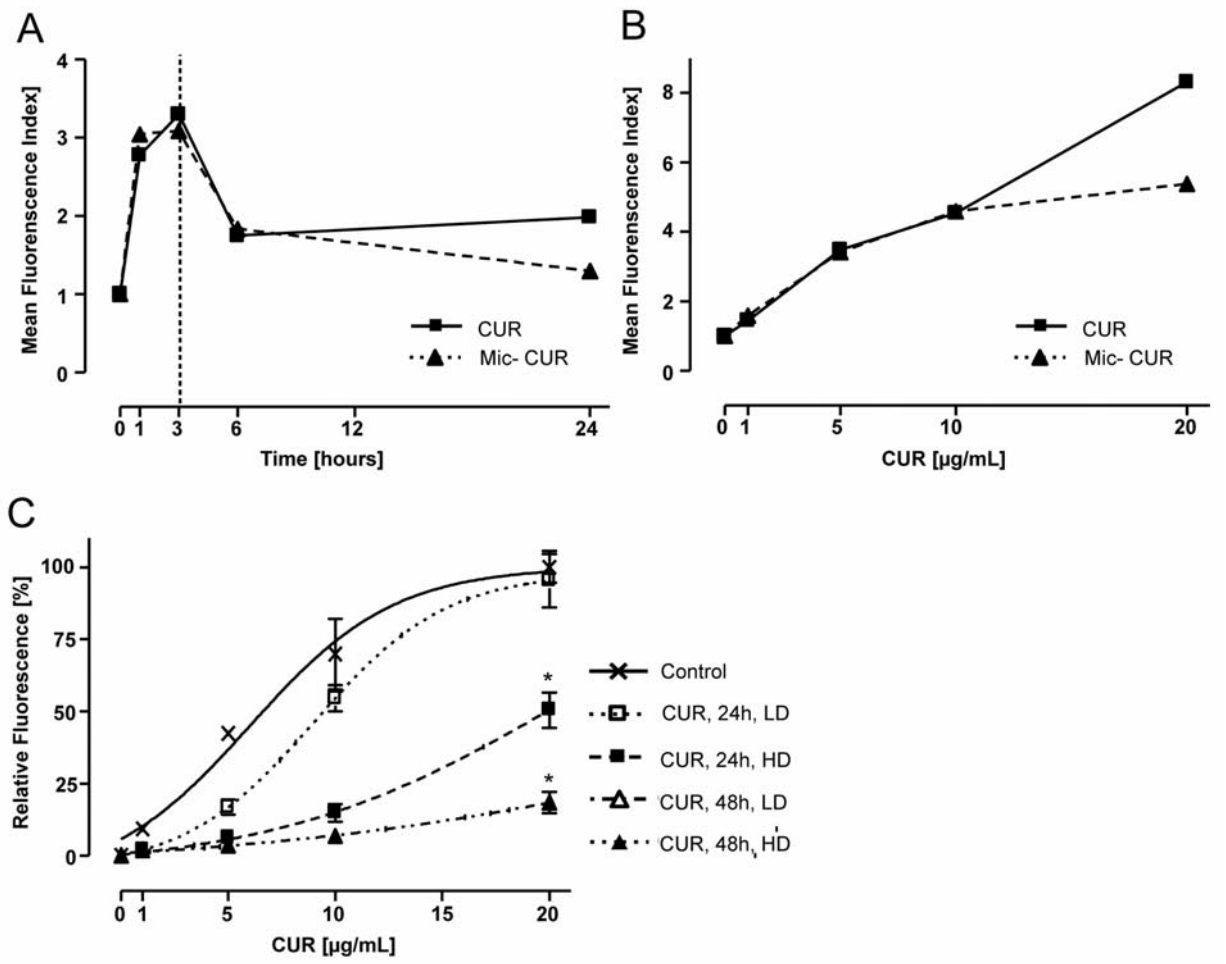

Figure 6. *Uptake and metabolisation of CUR by hepatoma cells. Example of HC-AFW1 cells. A. The intracellular uptake of CUR is highest during the first hour of incubation. The main decrease of intracellular CUR fluorescence occurs during the first 3 h after the CUR supply has stopped. B. With increasing CUR concentrations in the medium, the cellular uptake of CUR is rising. Concerning micellar CUR, the uptake is limited at around 10 µg/ml. C. The extracellular CUR fluorescence after CUR incubation is more reduced by high-density cultures compared to low-density cultures, *p<0.005. The difference between incubation periods (24 vs. 48 h) did not reach statistical significance. There are no significant differences between native and micellar CUR. The results for HUH6, HepT1 and HepG2 were similar.*

consecutive irradiation of the tumor bed with possible invisible micrometastases with blue light should take place shortly after curcumin administration. Another possible treatment option would be a local laparoscopic irradiation with blue light.

Under these circumstances, special interest is focused on the possible damages of a CUR-PDT combination to healthy tissues and cells. As we could show, together with PDT, CUR causes relevant loss of viability not only in hepatoma cells but also in fibroblast culture. In addition to this, possible damages to healthy liver tissue have to be ruled out; due to the high amount of cytochromes in liver cells, these cells are sensitive to PDT with the negative side-effect of cell damage in the healthy tissues next to the tumor. Without PDT, CUR alone (dark cytotoxicity) had very high IC_{50} to induce relevant loss of cell viability in fibroblasts. Furthermore, we have shown previously that the cellular concentrations after oral micellar CUR administration are somewhat higher in tumor cells compared to liver cells (12). *In vivo* analyses of PDT after CUR administration in an *in vivo* model will be the next step.

Conclusion

In summary, this *in vitro* study demonstrates that CUR-mediated PDT effectively enhances the anti-tumor properties of CUR in epithelial liver cancer cells (HC-AFW1, HUH6, HepT1 and HepG2) by inducing loss of viability *via* ROS

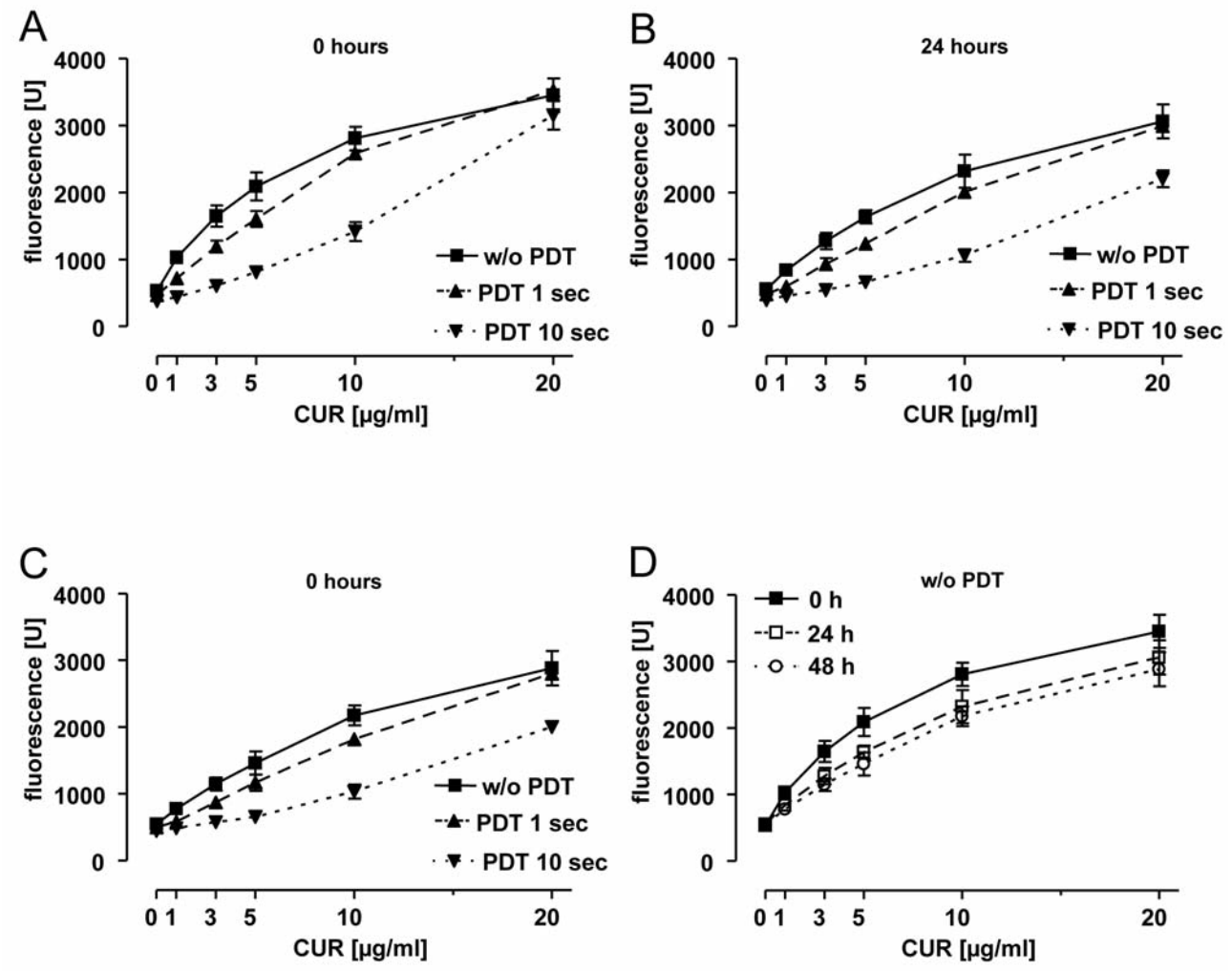

Figure 7. *The fluorescence of CUR is time-dependent and accelerated by exposure to blue light. A-C: Stronger degradation of CUR is seen after longer blue light exposure. D: Loss of fluorescence without blue light over time.*

production. Although further investigations are needed both *in vitro*, as well as *in vivo*, our data suggest that the use of PDT with CUR can be effective in the treatment of malignant epithelial liver tumors (HCC and HB).

Conflicts of Interest

The Authors state that they have no financial or non-financial competing interests concerning this manuscript.

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