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CARBOPLATIN RESISTANT HUMAN LARYNGEAL CARCINOMA CELLS ARE CROSS RESISTANT TO CURCUMIN DUE TO REDUCED CURCUMIN ACCUMULATION

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Abbreviations:

AO, acridine orange; BHT, butylated hydroxytoluene; BSO, L-buthionine-sulfoximine; DCF, 2′,7′-dichlorofluorescein; EtBr, ethidium bromide; GSH, glutathione, Hsp70, Heat shock protein 70; H_2DCFDA, 2′,7′-dichlorofluorescein diacetate; MDA, malondialdehyde; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NAC, N-acetyl-L-cysteine; PI, propidium iodide; PARP, poly(ADP-ribose)polymerase; PS, phosphatidylserine; ROS, reactive oxygen species.

Abstract

Curcumin is a natural compound that exhibits a wide range of beneficial effects, among them the anti-tumor activity. Recently it was shown that curcumin may be efficient against drug resistant tumor cells. The goal of our investigation was to examine if human laryngeal carcinoma cells resistant to carboplatin display sensitivity to curcumin, as compared to parental cells, and if this sensitivity is altered, to determine the molecular mechanisms that are responsible for it. We found that carboplatin resistant 7T cells were also cross resistant to curcumin. After the treatment with equimolar concentration of curcumin, 7T cells exhibited lower intracellular accumulation of curcumin which coincided with reduced formation of reactive oxygen species (ROS), diminished lipid and DNA damage followed by reduced induction of apoptosis and expression of heat shock protein 70 (Hsp70), as compared to parental HEp-2 cells. However, after the treatment with equitoxic concentration of curcumin, intracellular accumulation and all the explored downstream effects were similar in both cell lines suggesting that resistance of 7T cells to curcumin was based on its reduced intracellular accumulation. Since curcumin accumulates mostly in the membranes, we explored the fatty acid composition of both cell lines, but we did not find any difference between them.

Keywords: curcumin; drug resistance; ROS; lipid peroxidation; DNA damage; cellular accumulation

1. Introduction

Curcumin (diferuloylmethane) is a yellow pigment derived from the rhizome of the plant *Curcuma longa*. The powdered rhizome of this plant, called turmeric, has been used in traditional Chinese and Hindu medicine for centuries in the treatment of different diseases (Chattopadhyay et al., 2004). Although curcumin has shown a wide range of beneficial pharmacological effects, its anticancer properties have attracted a great interest. It has been shown that curcumin can induce the death of cancer cells and inhibit the formation of tumors in animal models of carcinogenesis (Kunnumakkara et al., 2008; Lopez-Lazaro, 2008; Teiten et al., 2010). Since curcumin influences multiple components of intracellular signaling pathways and molecular targets involved in the development and progression of cancer (Lopez-Lazaro, 2008; Teiten et al., 2010), it is a promising drug for the treatment of cancer as a multifactorial disease. The ongoing clinical trials should determine the possible beneficial effect of curcumin in cancer treatment, when given alone or in combination with conventional anticancer drugs (Teiten et al., 2010).

There are numerous reports about the production of reactive oxygen species (ROS) during curcumin-induced apoptosis in different tumor cell lines (Atsumi et al., 2005; Chen et al., 2010; Fujisawa S, 2004; Hail, 2008; Kang et al., 2005). These free radicals can cause serious damage to lipids, proteins, and DNA (Halliwell and Gutteridge, 1999) and they have been suggested as some of the initiators in apoptotic signaling (Carmody and Cotter, 2001; Circu and Aw, 2010). Unfortunately, tumor cells can often evade apoptosis through many different mechanisms. Overexpression of some antiapoptotic proteins like Bcl-2, Bcl-XL and Smac protected cancer cells from curcumin treatment (Karunagaran et al., 2005), while some curcumin resistant cell lines showed higher production of Hsp70 (Khar et al., 2001; Rashmi et al., 2003; Rashmi et al., 2004).

Resistance of tumor cells to chemotherapy is the major obstacle that limits the effectiveness of cancer treatment. Therefore, novel strategies based on new compounds that could be efficient against drug resistant tumor cells would be a promising way to increase the effectiveness of cancer treatment. Literature data suggest that curcumin exerts the same anticancer effects in several drug resistant cell lines as in their sensitive parental counterparts. This was shown for multidrug resistant myeloid leukemia cells (Lu et al., 2012), cisplatin resistant ovarian cancer cells (Weir et al., 2007), multiple myeloma cells resistant to

dexamethasone, doxorubicin and melphalan (Sung et al., 2009), as well as hormone independent, multidrug resistant breast cancer cells (Labbozzetta et al., 2009).

Drug resistance is a general problem in cancer treatment. Since curcumin has been shown to be very efficient as anticancer agent in some chemoresistant cell lines, the knowledge about the mechanisms involved in modified sensitivity of drug resistant cells to this natural compound could be important in future attempts of using curcumin as antitumor agent in clinical applications, especially in treatment of drug resistant cancers. The goal of our investigation was to examine if human laryngeal carcinoma cells resistant to carboplatin display sensitivity to curcumin, as compared to parental cells, and if this sensitivity is altered, to determine the molecular mechanisms that are responsible for it.

2. Materials and methods

2.1. Cell culture and treatment with curcumin

Human laryngeal carcinoma HEp-2 cells were obtained from cell culture bank (Gibco, Grand Island, NY). Development of their subline resistant to carboplatin has been published previously (Osmak et al., 1995). These cells were propagated for 26 passages without reversion in sensitivity to carboplatin and are now called 7T. HEp-2 and 7T cells were grown at 37ºC with 5% CO_2 as a monolayer culture in Dulbecco's modified Eagle's medium, DMEM (Gibco), supplemented with 10% newborn calf serum (Gibco). The doubling times for HEp-2 and 7T cells were 22 h and 26 h, respectively. Curcumin (Sigma, St. Louis, MO) was prepared in dimethylsulfoxide (DMSO) as 50 mM stock solution and stored at -20ºC for no longer than two months. After overnight incubation, cells were treated with curcumin in the dark (due to its photosensitivity) and diluted in culture medium (final concentration of DMSO being lower than 0.08%). After the treatment both adherent and floating cells were collected (adherent by tripsinization) centrifuged, counted and used in most of experiments, except for those in which the ROS induction was measured. In these experiments the cells were treated with curcumin in suspension.

2.2. Cytotoxicity assay

The sensitivity of cells to curcumin was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma) assay (Mickisch et al., 1990). Briefly, 2.500 HEp-2 cells and 3.500 7T cells were seeded in 180 μl medium in quadruplicate in 96-well

tissue culture plates and following overnight incubation treated with curcumin. After 72 h MTT was added and the absorbance of the formazan product was measured on a microplate reader (Awareness Technology Inc, Palm City, USA) at 600 nm. To examine the influence of ROS induction on survival of curcumin treated cells, they were pretreated for 1 h with 10 mM N-acetyl-L-cysteine (NAC; Sigma) and than co-treated with curcumin for additional 72 h. Survival of cells treated with both NAC and curcumin was calculated according to survival of cells treated only with NAC (with approximately 100% survival for both HEp-2 and 7T cells). In addition, cells were pretreated with 22.2 μg/ml L-buthionine-sulfoximine (BSO; Sigma) for 24 h and co-treated with curcumin for the next 48 h. Survival of cells treated with both BSO and curcumin was calculated according to survival of cells treated with BSO only (with the survival of more than 92% for both, HEp-2 and 7T cells).

Because the cytotoxic potency (as well as the intracellular accumulation) of a highly lipophilic curcumin *in vitro* depends on the composition of the *in vitro* system, i.e. the relation between cell number (volume) and medium volume and the binding protein and lipid content of the medium, we have done all our experiments in the medium supplemented with 10 % of newborn calf serum. In addition, we have examined the influence of cell number and the medium volume on the cytotoxicity of curcumin. As shown in Fig. S1, the alteration in cell number and in the medium volume that we used in our experiments, did not influence the cytotoxicity of curcumin. In addition, in order to have the same seeding density at the time of the treatment, we always kept the same ratio between the cell number and surface of the dish that we used.

2.3. Accumulation of curcumin

HEp-2 ($4x10^5$/10 ml medium) and 7T cells ($6x10^5$/10 ml medium) were seeded in 10 cm Petri dishes and after overnight incubation treated with curcumin for different time periods, collected, washed twice with cold PBS and held on ice until analysis. Due to the fact that curcumin exhibits a green fluorescent signal (Khopde et al., 2000; Wang et al., 2006), cells were analyzed using the FL1 channel of a FACSCalibur flow cytometer (Becton Dickinson; Franklin Lakes, NJ) equipped with laser emitting at 488 nm wavelength. Fluorescence emission (above 530 nm) from 10 000 cells was collected and analyzed with BD CellQuest Pro software (BD Biosciences, San Jose, CA).

2.4. ROS measurement

The intracellular accumulation of ROS was determined by flow cytometry using the 2´,7´-dichlorofluorescein diacetate probe (H_2DCFDA; Molecular Probes Invitrogen, Eugene, OR). H_2DCFDA behaves as a nonfluorescent molecule until acetate groups are removed by intracellular esterases. Following oxidation, it becomes a highly fluorescent dye 2´,7´-dichlorofluorescein (DCF). HEp-2 (1.2 x 10^6/12 ml medium) and 7T (1.7 x 10^6/12 ml medium) cells were seeded in 10 cm Petri dishes and following overnight incubation, they were collected, resuspended (2×10^5 cells/ml) in warm PBS without Ca^{+2} and Mg^{+2} ions and loaded with 20 μM H_2DCFDA for 30 min at 37ºC. After centrifugation, cells were suspended in 1 ml DMEM (supplemented with 10% newborn calf serum (Gibco) without phenol red (Gibco) and treated with curcumin for 15 minutes at 37ºC. Thereafter they were washed with PBS without Ca^{+2} and Mg^{+2} ions and the intracellular florescence of DCF was monitored by flow cytometry. Fluorescence emission above 530 nm was analyzed from 10000 cells. In each experiment three negative controls were used: a) untreated cells, in order to determine the contribution from autofluorescence, b) curcumin treated cells, in order to determine the contribution from curcumin fluorescence (similarly to DCF curcumin emits fluorescence above 530 nm) and c) cells treated with H_2DCFDA, in order to determine the level of intracellular ROS present before curcumin treatment.

2.5. Lipid peroxidation assay

Peroxidation of membrane lipids was determined by measuring the level of stabile and the most important final product of lipid peroxidation, malondialdehyde (MDA). MDA level was detected by HPLC after reaction with thiobarbituric acid. Briefly, HEp-2 (1.2 x 10^6/12 ml medium) and 7T (1.7 x 10^6/12 ml medium) cells were seeded in 10 cm Petri dishes and following overnight incubation treated with curcumin for 1 h, collected and washed twice with PBS. Supernatant was removed and cell pellets were placed at -80ºC. Afterwards cell pellets were sonicated 3 × 3 sec (Ultrasonic processor, Cole Parmer) on ice in the presence of 5 mM butylated hydroxytoluene (BHT; Sigma) in PBS (in cell concentration of 2×10^6 cells/75 μl PBS+BHT) to prevent sample oxidation. Then 60 μl of cell lysate were mixed with 400 μl of 0.1% H_3PO_4 and 100 μl of thiobarbituric acid and heated for 30 min at 100ºC. After heating, the samples were placed on ice to stop reaction. MDA level was determined on HPLC with fluorescent detector (set at λ_{ex} = 524 nm and λ_{em} = 551 nm). Concentration of MDA in the sample was quantified by peak-area measurement using the linear regression curve obtained for MDA standard solutions prepared from MDA standard (1,1,3,3-

tetramethoxypropane malonaldehyde bis(dimethyl acetal- acetal) (Sigma). Results were expressed as nmol/mg protein. Protein concentration in cell lysates was determined by the Bradford method.

2.6. DNA damage assay

HEp-2 (1.2 x 10^6/12 ml medium) and 7T (1.7 x 10^6/12 ml medium) cells were seeded in 10 cm Petri dishes and following overnight incubation treated with curcumin for 1 h, collected, washed with PBS and resuspended in ice-cold culture medium (2×10^5 cells/100 µl). Five microliters of suspension were mixed with 100 µl of 0.5% Low Melting Point agarose (Sigma) and analyzed by the alkaline comet assay (Singh et al., 1988). The formation of oxidized purines was determined using the formamidopyrimidine glycoslyase (Fpg) FLARE™ assay kit (Trevigen Inc, Gaithersburg, MD) with minor modifications (Gajski et al., 2008). Slides were analyzed at 250x magnification using an epifluorescent microscope (Zeiss, Göttingen, DE) connected trough a camera to an image-analysis system (Comet Assay II; Perceptive Instruments Ltd., Haverhill, Suffolk, UK). The comet parameters, tail length and tail moment, were used to measure the level of DNA damage. A total of 200 randomly captured nuclei were examined from each slide (100 nuclei from each duplicate slide) in two independent experiments.

2.7. Characteristic nuclear morphology of apoptotic cells

The nuclear morphology typical for apoptosis was determined 72 h after treatment with curcumin by epifluorescence microscopy (Axiovert 35, Opton). The cells were trypsinized, centrifuged and washed. Thereafter 10 µl of cell suspension were labeled with 4 µl of acridine orange (AO, 15 µg/ml in PBS) and 2 µl of ethidium bromide (EtBr, 50 µg/ml in PBS) and examined for typical nuclear morphology of apoptotic cells - the condensation of chromatin to compact and simple globular geometric figures. Both live and dead cells take up acridine orange. It intercalates into DNA, making it appear green, and binds to RNA, staining it red. Thus a viable cell will have bright green chromatin in its nucleus and red-orange cytoplasm. Ethidium bromide is only taken up by nonviable cells. It intercalates into DNA, making it appear orange, but binds only weakly to RNA, which may appear slightly red. Thus a dead cell will have bright orange chromatin (the ethidium overwhelms the acridine) and its cytoplasm, if it has any contents remaining, will appear dark red. Cells that have undergone necrosis will have the fluorescent features of nonviable cells but will not have apoptotic nuclear morphology. Using this method, both normal or apoptotic nuclei in live cells will

fluoresce bright green. In striking contrast, apoptotic nuclei in dead cells will fluorescence bright orange. Fluorescence was detected through the BP 450-490, FT 510, LP 520 filter. The images were taken with the camera Pixera Pro150ES.

2.8. Annexin V-FITC/PI assay

The appearance of phosphatidylserine (PS) on the extracellular side of membrane, as a marker of apoptosis, was evaluated with Annexin V-FITC/PI method as described previously (Jakopec et al., 2006). The percentages of three cell death categories were determined: apoptotic (AV+, PI-), and secondary necrotic or necrotic (AV+, PI+) and live cells (AV-, PI). Annexin V-FITC was obtained from BD Pharmingen (BD Biosciences, San Jose, CA).

2.9. Western blot

Cells were seeded and treated with curcumin on the next day. Protein extracts of control and treated cells were prepared by lysing 7.5×10^5 cells in 100 µl of 1 × SDS sample buffer (0.0625 M Tris HCl, pH 6.8, 2% SDS, 10% glycerol, 0.01% bromphenol blue, 5%(v/v) β-mercaptoethanol). Samples were then sonicated, denaturated by boiling in water bath for 1 min, loaded on 12.5% SDS-polyacrylamide gel, electrophoresed, and transferred to nitrocelulose membrane (0.22 µm; Protran, Schleicher & Schuell, DE). Membrane was blocked with blocking buffer (TBS containing 0.1% Tween 20 (v/v) (Sigma) and 5% milk (w/v) (Sirela, Zagreb, HR) for 1 hour at the room temperature. Primary antibodies (applied for 1 hour at room temperature or overnight at 4 °C) were: anti-procaspase-3 (sc-7272, Santa Cruz) and anti-PARP (556362, Pharmingen). They were diluted 1:1000 (procaspase-3) or 1: 4000 (PARP). Thereafter, membranes were incubated for 1 h with HRP-labeled sheep anti-mouse secondary antibody diluted 1:5000 (NA 931V, Amersham Pharmacia Biotech, Uppsala, SE) and then developed by an ECL system according to the manufacturer's instructions (PerkinElmer, Waltham, MA).

2.10. Determination of fatty acid methyl esters

HEp-2 and 7T cells were grown in 75 cm^2 flasks for 48 h until they reached 80% confluence. Thereafter, 1.5×10^7 cells were collected by trypsinization, washed with 0.9% NaCl and lyzed in 100 µl of dH_2O. Total lipids were extracted from lysate with a mixture of isopropanol/chlorophorm (1,5:1 v/v) (Rose and Oklander, 1965). Fatty acids were transesterified to methyl esters with 2 ml of 1 M HCl in methanol at 100^oC for 45 min. Fatty acid methyl esters were extracted twice with petrol-ether and analyzed by a SRI 8610C GAS

CHROMATOGRAPH (SRI Instruments Chromatography Systems, Torrance, CA), equipped with a flame ionization detector and a capillary column 007-CW (Quadrex Corporation, Woodbridge, USA), length 60 m, i. d. 0.25 mm, and film thickness 0.25 μm. Hydrogen was used as a carrier gas with a flow of 60 ml/min in a split mode. The initial oven temperature of 150^{o}C was maintained for 3 min, than increased to 220^{o}C at a rate of 8^{o}C/min, and the final temperature was kept constant for 30 minutes. The injector and detector temperatures were 250^{o}C and 260^{o}C, respectively. Fatty acid methyl esters were identified by comparison of their retention times to those of known reference standards (Supelco Inc., Bellefonte, PA). The results were collected and analyzed using Peak Simple 3D, Version 2.97 (SRI Inc., Torrance, CA).

2.11. Statistical analysis

Data were analyzed by unpaired Student's t-test and expressed as means ± standard deviation. Results of the comet assay parameters are presented as means ± standard error, and were evaluated using Statistica 5.0 program package (StaSoft, Tulsa, OK). In order to normalize the distribution and equalize the variances of the comet assay data, a logarithmic transformation was applied. Multiple comparisons between groups were done by means of ANOVA on log-transformed data. Post-hoc analyses of differences were done by the Newman-Keuls test. Data were considered statistically significant at p-value of < 0.05.

3. Results

3.1. Sensitivity of HEp-2 and 7T cell line to curcumin

Human laryngeal carcinoma HEp-2 cells and the carboplatin resistant 7T subline were exposed to various concentrations of curcumin, and their survival was determined 72 h later. Curcumin inhibited the growth of both cell lines in a dose-dependent manner, but carboplatin resistant 7T cells were cross resistant to this natural drug (Fig. 1). From obtained survival curve we selected two concentrations of curcumin, equimolar and equitoxic, for further studies. At equimolar concentration of 25 μM curcumin the differences in cell survival between HEp-2 and 7T cells were most pronounced: 30% percent of HEp-2 cells survived this curcumin concentration, as compared to 65% of 7T cells. To reach the same survival level, 7T cells had to be treated with 40 μM curcumin and this concentration was marked as equitoxic concentration.

3.2. Curcumin accumulation in HEp-2 and 7T cells

We quantified curcumin accumulation by flow cytometry since curcumin has the ability to fluoresce in the green band. Curcumin fluorescence is weak in aqueous solutions (Khopde et al., 2000), but in hydrophobic micelles it is enhanced by nearly 55 times (Wang et al., 2006). Since curcumin is a lipophilic compound and accumulates mainly in the membranes (Kunwar et al., 2008), its accumulation in hydrophobic parts of membranes probably explains significant fluorescence that was detected by flow cytometry. The presence of extracellularly bound curcumin is probably not detected due to its weak florescence in aqueous solutions.

Since 50% of curcumin degrades in cell culture medium after 8 h of incubation (Wang et al., 1997), we have chosen 1.5 and 3 hour period to measure cellular accumulation of curcumin. The results of flow cytometry measurements showed that curcumin accumulation in both cell lines is concentration dependent and has the same dynamic. In both cell lines the maximum of intracellular curcumin fluorescence was achieved after 1.5 h. However, 7T cell line accumulated less curcumin at equimolar curcumin concentration as compared to HEp-2 line, while at equitoxic concentration the accumulation was similar (Fig. 2A). According to the measurements on Beckman Cell Counter (data not shown), and also on flow cytometric histograms of cell size distribution represented by Forward Scatter-Height parameter (FSC-H), we detected smaller average cell size of 7T cells as compared to HEp-2 cells (Fig. 2B). Since cell size can influence the amount of accumulated curcumin, it could be possible that 7T cells accumulate less curcumin due to their smaller average cell size. To see if the accumulation of curcumin in 7T cells is also decreased in comparison to HEp-2 cells if only the cells of the same size were compared, we created a new approach to analyze flow cytometric data. For this purpose we set four gates in FSC-H histogram of both cell lines treated with curcumin (R1, R2, R3 and R4) (Fig. 2B) that encompassed cells with increasing cell size, and for each cell size the amount of accumulated curcumin was determined. The results (Fig. 2C) indicate that in 7T cell line treated with 25 μM curcumin no positive correlation for curcumin accumulation and cell size was observed, but for 40 μM curcumin this correlation existed, i.e. bigger cells accumulated more curcumin. For HEp-2 cell line this correlation was present at both concentrations of curcumin. In 7T cells lower accumulation of curcumin was observed at both gates applied (R2 and R3), although this difference was statistically significant only for the cells with bigger size (R3 gate). Also, at equitoxic concentrations HEp-2 and 7T cells of the same size accumulated the same amount of

curcumin (Fig. 2C see R3 gate at 25 μM for HEp-2, and R3 gate at 40 μM for 7T). Therefore, 7T subline accumulates less curcumin than HEp-2 cell line, and this difference in not caused by the different size of the cells in these two populations.

3.3. Curcumin induced ROS formation in HEp-2 and 7T cells

Numerous literature data suggest that ROS induction is one of the first effects of curcumin treatment and it is involved in its cytotoxicity (Chen et al., 2010; Fujisawa S, 2004; Khar et al., 2001). Therefore, we examined and compared ROS induction in HEp-2 and 7T cells following the treatment with curcumin. Using H_2DCFDA probe, ROS induction was explored by flow cytometry. Results presented in Fig. 3A show that after only 15 minutes of treatment with curcumin the induction of ROS was different in HEp-2 *versus* 7T cells. The level of ROS significantly increased in HEp-2 cells, but only slightly in 7T cells for both, equimolar and equitoxic concentrations (Fig. 3A). To further examine the role of ROS induction in differential sensitivity of HEp-2 and 7T cells to curcumin, the cells were pretreated with NAC or BSO. NAC can directly scavenge free radicals, but it is also involved in glutathione (GSH) synthesis. BSO inhibits the synthesis of GSH, and in this way reduces the level of antioxidant GSH molecules. After the treatment with equimolar (25 μM) concentration of curcumin, the results of MTT assay showed less influence of NAC on survival of 7T cells than HEp-2 cells (Fig. 3B). If the cells were pretreated with BSO, again the differences in cell survival were more expressed in HEp-2 cells (Fig. 3C). However, the influence on cell survival of both, NAC and BSO was similar on HEp-2 and 7T cells when equitoxic concentration of curcumin was used (Fig. 3B and 3C) thus indicating that at this concentration in both cell lines ROS induction was the same. The possible explanation for the discrepancy between MTT data for equitoxic ROS induction (the experiments with NAC and BSO) and flow cytometry data (the experiments with direct ROS measurement) could lie in the fact that different experimental time points were examined. While ROS induction was measured by flow cytometry after only 15 min of treatment with curcumin, because for longer incubation time the leakage of H_2DCFDA from both cell lines was observed, in MTT experiments treatment with curcumin in combination with NAC and BSO lasted much longer. Thus, although the survival of both HEp-2 and 7T cells pretreated with NAC or BSO was similar for equitoxic doses of curcumin, suggesting that the same amount of ROS was induced, at the same dose HEp-2 and 7T cells showed clear difference in the early ROS induction.

3.4. Curcumin induced lipid and DNA damage in HEp-2 and 7T cells

ROS can react with most of cellular macromolecules including lipids and DNA (Kohen and Nyska, 2002). Therefore, after we observed that early induction of ROS was different in HEp-2 *versus* 7T cells following the curcumin treatment, we decided to examine and compare downstream consequences of ROS formation. Results of HPLC analysis of MDA level, which is one of the major final products of lipid peroxidation, showed that curcumin induced lipid peroxidation in both cell lines. The level of MDA correlated with cytotoxicity of curcumin: at equimolar concentration of curcumin (25 μM), significantly more MDA was determined in HEp-2 cells than in 7T cells, but at equitoxic concentration (40 μM) the amount of MDA was almost the same (Fig. 4A).

Oxidative damage at DNA level was detected by the FPG-modified comet assay. The results showed that in both cell lines there was significant increase in DNA strand breaks (Fig. 4B, see buffer control), but only in HEp-2 cell line a small increase in oxidative DNA damage was detected at both concentrations of curcumin used or just for equitoxic dose by the parameter of tail moment (Fig. 4B). The presence of DNA strand breaks was also confirmed by standard alkaline comet assay (Fig. 4C), which again showed correlation with cytotoxicity of curcumin and equitoxic doses showed the similar amount of DNA strand breaks.

3.5. Curcumin induced apoptosis in HEp-2 and 7T cells

In order to see the downstream effects of curcumin induced ROS formation, lipid and DNA damage in HEp-2 and 7T cells, we applied different techniques. The results presented in Fig. 5A show typical nuclear apoptotic characteristics (condensation of chromatin to compact and simple globular geometric figures) in HEp-2 and 7T cells after the treatment with curcumin. Further, phosphatidylserine was exposed on outer side of membrane (Fig. 5B), PARP was cleaved and caspase-3 activated (Fig. 5C). All these results indicate that curcumin treatment induced apoptotic cell death in HEp-2 and 7T cells. At equimolar concentration of curcumin, less apoptotic cells was found in 7T cells (Fig. 5A and 5B); this was confirmed by lack of cleavage of PARP and procaspase-3 (Fig. 5C). However, at equitoxic concentration apoptosis induction was similar in both cell lines (Fig. 5B and 5C).

3.6. Curcumin induced Hsp70 expression in HEp-2 and 7T cells

It was found that curcumin induces Hsp70 expression in different cell lines and thereby protects them from apoptotic cell death (Khar et al., 2001). Therefore we examined the induction of Hsp70 following the treatment with curcumin. As shown in Fig. 5D, at equimolar

concentration of curcumin induction of Hsp70 expression was lower in 7T cells, as compared to HEp-2 cells, but at equitoxic concentration the induction was similar. Thus it can be concluded that Hsp70 expression is proportional to the curcumin induced stress detected in both cell lines.

3.7. Fatty acid composition of HEp-2 and 7T cell membranes

Curcumin is a lipophilic compound and accumulates mainly in the membranes. To explain the reduced accumulation of curcumin in 7T cells, we decided to explore membrane fatty acid composition. The results of gas chromatography presented in Fig. 6 show that there was no difference in fatty acid composition in 7T subline compared to HEp-2 cells.

4. Discussion

In order to increase the effectiveness of cancer treatment, the interest has been recently focused to the drugs that have been used in traditional medicine (Chattopadhyay et al., 2004), like curcumin. The observed anti-tumor effects of this natural compound encouraged extensive efforts to establish its usefulness in cancer prevention and cancer treatment. Curcumin could be an excellent candidate to target a multifactorial disease like cancer because of its broad range of activities, based on its ability to affect multiple intracellular targets (Kunnumakkara et al., 2008; Lopez-Lazaro, 2008; Teiten et al., 2010).

A particular problem in classic chemotherapy is the drug resistance of tumor cells. The drugs that could be efficient against chemoresistant cells could be of great importance in enhancing effectiveness of cancer treatment. There are some data in the literature that verified anticancer properties of curcumin in drug resistant cells (Labbozzetta et al., 2009; Lu et al., 2012; Sung et al., 2009; Weir et al., 2007). However, these data should be more abundant in order to make more general conclusions about curcumin's activity against drug resistant cells. To our knowledge, such data are missing for laryngeal carcinoma cells. Therefore our study was focused on carboplatin resistant human laryngeal carcinoma cells. Our aim was to explore their sensitivity to curcumin, and if this sensitivity is altered, to determine the molecular mechanism that are responsible for it. We found that carboplatin resistant 7T cells are also cross resistant to curcumin.

Curcumin is a lipophilic compound, unstable in aqueous solutions (Wang et al., 1997), In the study on rat thymocytes it was shown that such properties enable this molecule to easily

enter the cell and localize in the membrane regions like: plasma membrane, membranes of endoplasmic reticulum and nuclear envelope (Jaruga et al., 1998). In one of the recent papers it was found that, except for membrane localization, minor nuclear localization occurred in mouse spleen lymphocytes, mouse fibroblast (NIH3T3), T cell lymphoma of murine origin (EL4) and breast cancer cell line of human origin (MCF7) (Kunwar et al., 2008). Cytotoxicity study on these cell lines indicated that curcumin toxicity in one individual cell line increased with its increased accumulation, but general correlation between accumulation and toxicity was not found. To see if there is some correlation between accumulation and cytotoxicity in our model system, curcumin accumulation was explored based on quantifying the fluorescence of intracellular curcumin. Our results showed that 7T cells accumulate less curcumin compared to HEp-2 cells. Since intracellular curcumin accumulation was similar in both cell lines at equitoxic doses, we supposed that reduced cytotoxicity of curcumin applied in equimolar doses could be due to lower intracellular accumulation of curcumin in 7T cells. To confirm our hypothesis, we explored the downstream events of curcumin accumulation.

There are numerous reports about the production of reactive oxygen species (ROS) as one of the first steps in curcumin cytotoxicity to different tumor cell lines (Atsumi et al., 2005; Chen et al., 2010; Fujisawa S, 2004; Hail, 2008; Kang et al., 2005). We also detected ROS formation quite early after curcumin was added and found more ROS in parental HEp-2 cells. ROS presence and its role in cytotoxicity were also confirmed for both HEp-2 and 7T cell line after longer period of incubation with curcumin combined with NAC and BSO treatment. Since both of them influence cellular GSH level, we checked basal GSH concentration in both cell lines and found that it was the same (data not shown). Although combined treatment of curcumin with NAC or BSO affected the survival of HEp-2 and 7T cells, their influence on equitoxic doses of curcumin was similar, suggesting that the same amount of ROS was induced after longer incubation time. Due to discrepancies in results about ROS amount in 7T cells during short and longer incubation time with equitoxic dose of curcumin, we concluded that ROS induction was probably lower in the beginning of the treatment (15 min) in the 7T cell line. Contribution to lower early induction of ROS could be due to lower accumulation of curcumin detected in 7T cells at the same time (which was measured as negative control in ROS experiments) (Fig. S2). But, after longer incubation times with equitoxic concentration of curcumin, both cell lines accumulated the same amount of curcumin and also impact of ROS on the cytotoxicity was the same. Therefore, we concluded that when the same amount of curcumin was accumulated in both cell lines, the same amount of ROS was formed. However, early difference in ROS formation and curcumin

accumulation detected in 7T cells could have some influence on altered response of 7T *versus* HEp-2 cells to curcumin.

ROS can cause serious damage to lipids, proteins, and DNA (Halliwell and Gutteridge, 1999). Since we detected an early alteration in ROS formation following the treatment with curcumin, we also measured early ROS induced damage on lipids and DNA. Our results showed that curcumin treatment in HEp-2 and 7T cell line induced lipid peroxidation and DNA damage after only one hour of treatment. The level of lipid peroxidation correlated with cytotoxicity of curcumin. Similar correlation between curcumin induced ROS formation, lipid damage and cytotoxicity was observed on primary gingivial fibroblast (HGF) and human submandibular adenocarcinoma cells (HSG) (Atsumi et al., 2005).

The induction of DNA damage that we observed in HEp-2 and 7T cells following the treatment with curcumin is in agreement with those previously reported, despite differences in the type of cells and experimental conditions used (Cao et al., 2006; Huang et al., 2011; Jiang et al., 2010; Lin et al., 2008; Lopez-Lazaro et al., 2007; Su et al., 2006; Urbina-Cano et al., 2006). Although we have expected that more oxidative DNA damage (like oxidized purines) would appear in HEp-2 and 7T cells because of ROS induction, strand breaks were predominant and correlated with cytotoxicity. However, minor oxidative DNA damage was found only in HEp-2 cells, and this could be the result of enhanced early ROS induction found in these cells. Presence of DNA strand breaks after curcumin treatment could be explained by the work of Jiang et al (2010). They showed the role of mismatch repair system in curcumin induction of DNA double strand breaks at the place of mutagenic adduct 8-oxo guanine. Also, curcumin has been shown to induce topoisomerase I- and II-DNA complexes in K562 leukemia cells (Lopez-Lazaro et al., 2007). They were inhibited by the antioxidants like NAC, suggesting that their formation was mediated by ROS. Cellular processing converts these complexes into permanent DNA strand breaks that trigger cell death (Binaschi et al., 1995; Champoux, 2001; Onda et al., 2008; Sordet et al., 2003).

There are numerous reports about ROS involvement in curcumin induced apoptosis in the literature (Atsumi et al., 2005; Chen et al., 2010; Fujisawa S, 2004; Hail, 2008; Kang et al., 2005). Apoptosis inhibition was found to be one of the causes of resistance to curcumin in some cell lines (Karunagaran et al., 2005). Therefore, we decided to explore curcumin induced cell death in HEp-2 and 7T cells. The results of different methods showed that curcumin treatment induced apoptotic cell death in both cell lines which was in correlation with curcumin cytotoxicity. When intracellular accumulation of curcumin, ROS induction,

and the level of lipid and DNA damage were similar in HEp-2 and 7T cells (as observed for equitoxic doses of curcumin), the downstream effect, i. e. apoptosis induction, was also similar. Therefore, we excluded the alterations in apoptosis induction as possible cause of curcumin resistance in 7T cell line. On the contrary, several reports showed that inhibition of apoptosis was the cause of curcumin resistance. For example, a number of published data reported that overexpression of antiapoptotic proteins, like Bcl-2, Bcl-XL and Smac protected cancer cells from curcumin treatment (Karunagaran et al., 2005). Therefore we examined the expression of antiapoptotic Bcl-2 protein. We determined its expression until the 24 h after the treatment when apoptosis was detected by PARP cleavage (Fig. S3). There were no increase in Bcl-2 expression neither in HEp-2 nor 7T cell line during that time period (Fig. S2). This further supported our conclusion that altered expression of antiapoptotic proteins is not the cause of curcumin resistance in 7T cell line.

Hsp70 is usually expressed upon different stress stimuli (Silver and Noble, 2011). Curcumin resistant cell lines established from lung, kidney, prostate, cervix, CNS malignancies and melanomas showed higher production of antiapoptotic protein Hsp70 upon ROS induction and thereby protecting them from apoptotic cell death (Khar et al., 2001). Also, human colon cancer cells treated with curcumin were protected from apoptotic cell death due to Hsp70 induction upon heat treatment (Rashmi et al., 2003) or ectopic Hsp70 overexpression (Rashmi et al., 2004). Hsp70 silencing enhanced curcumin induced apoptosis in those cancer cells (Rashmi et al., 2004). Therefore, we examined the expression of Hsp70 in HEp-2 and 7T cells following the treatment with curcumin. At equimolar concentration of curcumin the induction of Hsp70 expression was lower in 7T as compared to HEp-2 cells. But at equitoxic concentration, the Hsp70 induction was similar in both cell lines. Therefore, we can conclude that Hsp70 expression correlates with the level of stress detected in both cell lines. However, if Hsp70 induction had some antiapoptotic role during the curcumin treatment, its influence was similar in both, curcumin sensitive HEp-2 and resistant 7T cell line.

Changes in drug accumulation can play an important role in drug sensitivity and drug resistance of tumor cells (Casas et al., 2011; Fodale et al., 2011; Gottesman, 2002). This can be achieved through altered properties in cell membrane lipids as it was recently shown with isolated lipids from doxorubicin sensitive (MCF-7) and resistant (MCF-7/ADR) breast cancer cells (Peetla et al., 2010). Also, fatty acid analysis of vincristine sensitive KB-3-1 and vincristine resistant KB-ChR-8-5 cells showed that the resistant cell line had lower amounts of unsaturated fatty acids (Das et al., 1998). In line with that, when membrane phospholipids

of L1210 murine leukemia cells were enriched with fatty acids of variable saturation degrees the accumulation of adriamycin directly correlated with the degree of fatty acid membrane saturation (Burns and North, 1986).

In order to determine the cause of reduced curcumin accumulation in 7T cells, accompanied by its lower cytotoxicity in these cells, we explored some characteristics of cell membranes in HEp-2 and 7T cell line, since curcumin is a lipophilic compound and accumulates mainly in the membranes. Our results showed that there was no difference in membrane fatty acid composition between 7T and HEp-2 cells. We must point out that in our experiments we extracted lipids from whole cells, because curcumin can accumulate not only in plasma membrane, but also in membranes of endoplasmic reticulum and nuclear envelope (Jaruga et al., 1998). Therefore, we can not exclude some differences in fatty acid composition between membranes of different cell compartments. An additional cause of changed intracellular curcumin accumulation could be the altered activity of ATP hydrolyzing cellular transporters, a superfamily of transmembrane proteins, that export a wide variety of natural and synthetic compounds from the cells (Deeley et al., 2006). One of them, the ATP-binding cassette transporter ABCA1, is involved in cholesterol efflux, but can also influence the sensitivity to curcumin, as shown recently (Bachmeier et al., 2009). To further explore the molecular mechanisms of altered curcumin accumulation in drug resistant 7T cells, our future research will be focused on cell membranes and membrane transporters.

In conclusion, natural product curcumin derived from plant *Curcuma longa* has shown a wide range of beneficial effects as antitumor agent. Although curcumin exhibits promising potential in treatment of chemoresistant cancer cells, we report here for the first time that drug resistant cells can become also cross resistant to curcumin, as we found for carboplatin resistant laryngeal carcinoma cells. Reduced curcumin accumulation was one of the possible explanations for curcumin resistance. The knowledge about the mechanisms involved in modified sensitivity of drug resistant cells to curcumin could be important in the future attempts of clinical application of curcumin as antitumor agent, especially in treatment of chemoresistant cancers. Our results indicate that the effects of curcumin in the potential treatment of drug resistant tumors may depend on tumor type or drug that was involved in drug resistance development. Since curcumin is a lipophilic molecule, which leads to problems of decreased solubility and bioavailability, intensive research is currently being conducted in the field of curcumin delivery. This could be crucial in future attempts of overcoming curcumin resistance based on diminished cellular accumulation like the one observed in carboplatin resistant 7T cell line.

Conflict of interest

All authors declare that they have no conflict of interest.

Aknowledgements

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FIGURE CAPTIONS

Figure 1. Survival of human laryngeal carcinoma HEp-2 cells and their derived carboplatin resistant 7T subline following the treatment with curcumin. HEp-2 and 7T cells were incubated for 72 h with different concentrations of curcumin and their survival was determined by MTT assay. The results are shown as mean values of three experiments (± S.D.). * $p<0.01$ and ** $p<0.001$ versus HEp-2.

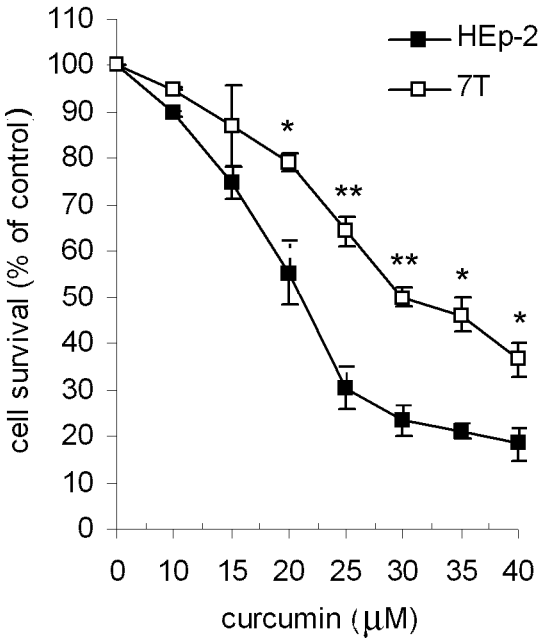

Figure 2. Impact of incubation time and cell size on the accumulation of curcumin in HEp-2 and 7T cells. A) Cells were incubated with 25 and 40 μM curcumin, harvested by trypsinization after indicated time intervals, washed twice with PBS, and intracellular curcumin fluorescence was analyzed on FACSCalibur flow cytometer. In the presented data the autofluorescence of untreated cells (MFI less than 5) was subtracted from the mean fluorescence intensity (MFI) of intracellular curcumin in treated cells. The results are shown as mean values of three experiments ± S.D. * $p<0.05$ versus HEp-2. (B) Representative histogram of cell size distribution in HEp-2 and 7T cell population after 3 h treatment with the indicated concentrations of curcumin. R1, R2, R3 and R4 gates represent cells of different size (each gate represents 5-15% of the entire population of analyzed cells). (C) Mean values of intracellular curcumin MFI in HEp-2 and 7T cells of different size. The results are shown as mean values of three independent experiments (± S.D.). * $p<0.05$ versus R2 gate of HEp-2; ** $p<0.05$ versus R1 gate of 7T; *** $p<0.05$.

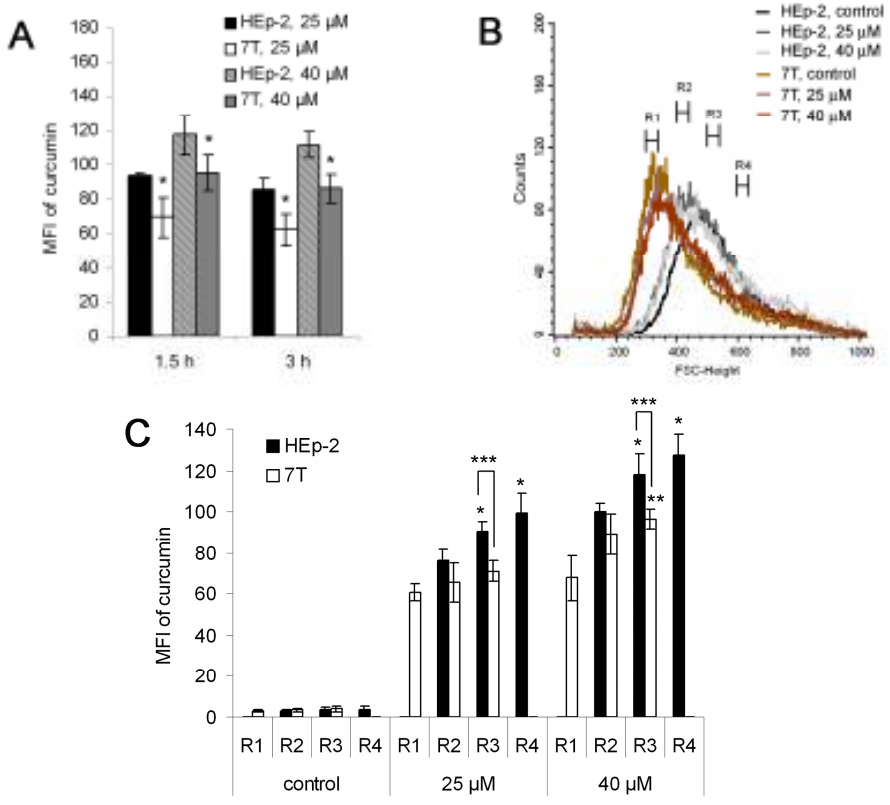

Figure 3. Induction of ROS in HEp-2 and 7T cells following the treatment with curcumin. A) Cells were pretreated for 30 min with 20 μM H2DCFDA at 37ºC, washed, and incubated for 15 min with 25 and 40 μM curcumin. Intracellular DCF (as the measure of ROS) and curcumin fluorescence were analyzed on FACSCalibur flow cytometer. FL1 histograms of HEp-2 and 7T cell population are shown as representative from three independent experiments. B) Impact of NAC on cell survival following the treatment with curcumin. Cell survival was determined by MTT assay. NAC (10 mM) was added 1 h before curcumin and it was present during 72 h treatment with curcumin. Increase in cell survival after the treatment with NAC (the number above the connections between two bars) indicates the mean value of two experiments. It is calculated by dividing precentage of cell survival after curcumin treatment with NAC pretreatment and survival after curcumin treatment alone. C) Impact of BSO pretreatment on survival of cells exposed to curcumin, as determined by MTT assay. Cells were pretreated with BSO (22.2 μg/ml) for 24 h and then treated with curcumin (with BSO) for the next 48 h. Decrease in cell survival (the number above the connections between two bars) after the treatment with BSO indicates the mean value of two experiments. It is calculated by dividing precentage of cell survival after curcumin treatment alone and survival after curcumin treatment with BSO pretreatment.

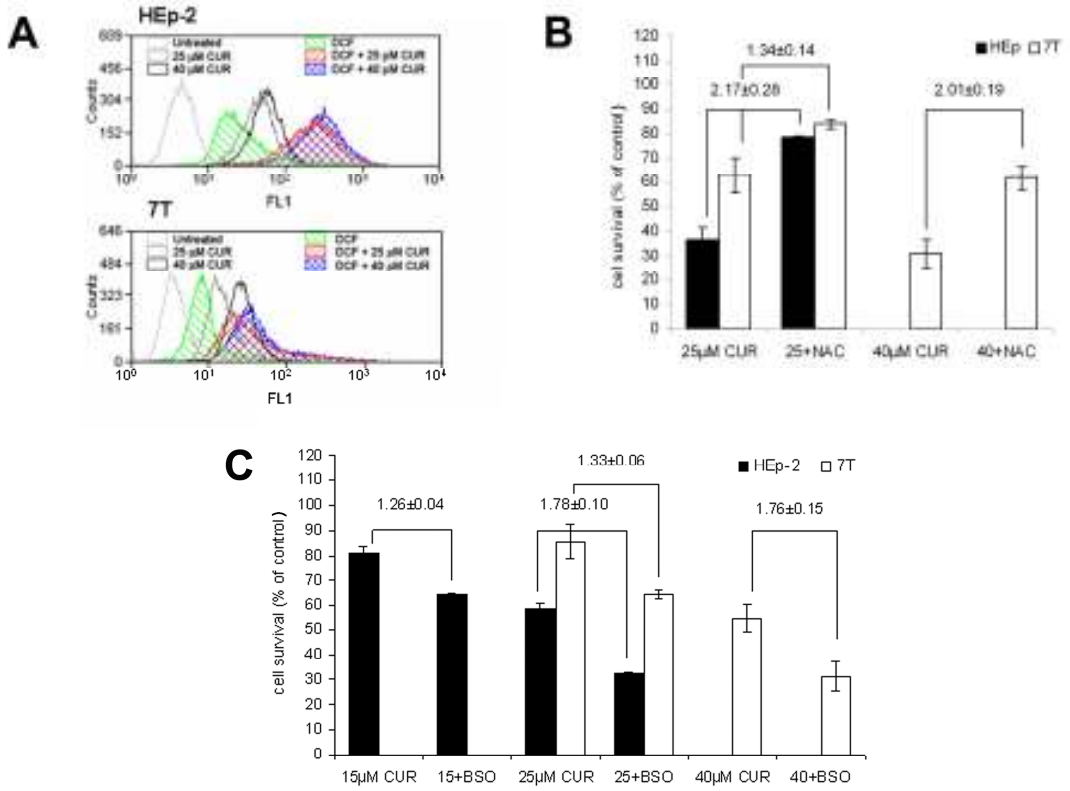

Figure 4. Induction of lipid and DNA damage during curcumin treatment of HEp-2 and 7T cells. A) After 1 h treatment with curcumin, cell lysates were analyzed by HPLC and the concentration of MDA, as lipid peroxidation product, was determined. The results are shown as mean values of two independent experiments . For detection of DNA damage comet assay was used and total of 200 randomly captured nuclei were examined from each slide (100 nuclei from each duplicate slide). Tail length and tail moment were used to measure the level of DNA damage. B) FPG comet assay was performed for detection of oxidative DNA damage after 1 h treatment with curcumin. * $p<0.05$ versus buffer. C) Alkaline comet assay was performed for detection of DNA strand breaks after 1 h treatment with the indicated concentrations of curcumin. * $p<0.05$.

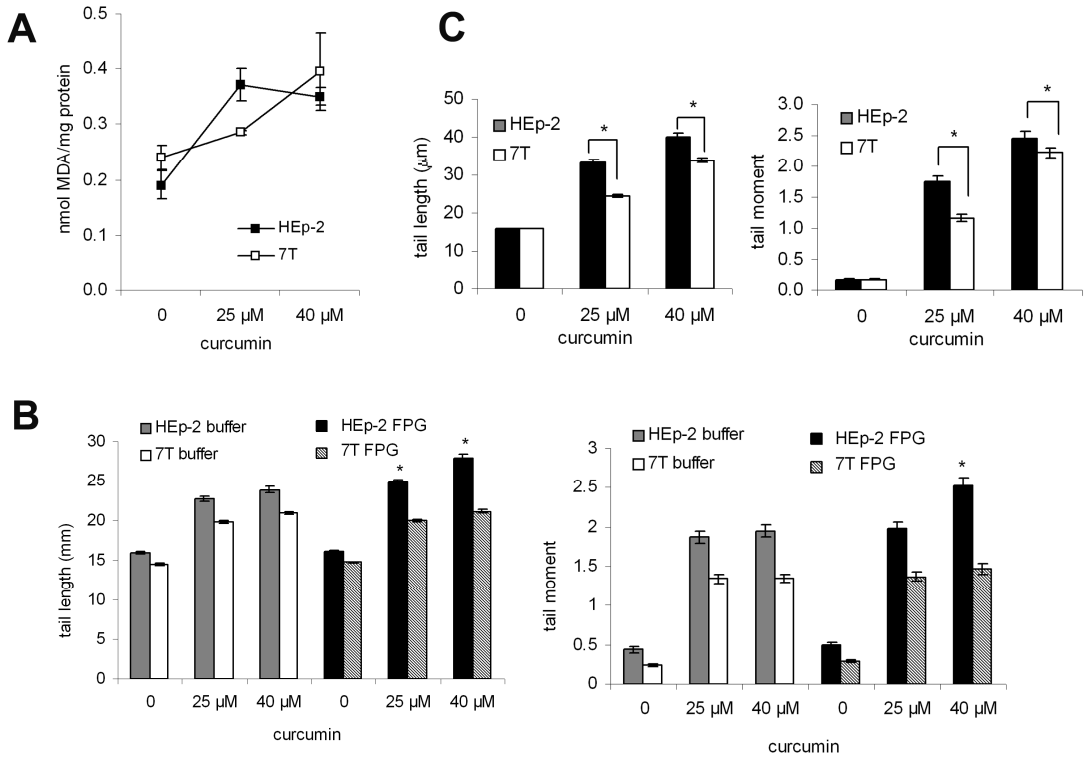

Figure 5. Apoptosis induction and Hsp70 expression in HEp-2 and 7T cells after curcumin treatment. A) Apoptotic nuclear morphology of HEp-2 and 7T cells stained with acridin orange and ethidium bromide after 72 h treatment with curcumin. Pictures were taken at magnification 1000 x. B) Using the the appearance of phosphatidylserine (PS) on the extracellular side of membrane, evaluated with AnexinV-FITC/Propidium iodide flow cytometry method, the induction of cell death in 10.000 cells was analyzed after 72 h treatment with curcumin. The percentages of three cell death categories were determined: apoptotic (AnexinV positive (AV+), propidium iodide negative (PI-), and secondary necrotic or necrotic (AV+, PI+), as well as and live cells (AV-, PI-). Graph shows representative values of two independent experiments. C) Western blot analysis of PARP and procaspase-3 cleavage as markers of apoptotic cell death. HEp-2 and 7T cells were treated for 72 h with the indicated concentrations of curcumin. Membrane stained with Ponceau S was used as loading control. Representative of three independent experiments is shown. D) Western blot analysis of Hsp70 expression after 24 h treatment with curcumin. Membrane stained with Ponceau S was used as loading control. Representative of three independent experiments is shown.

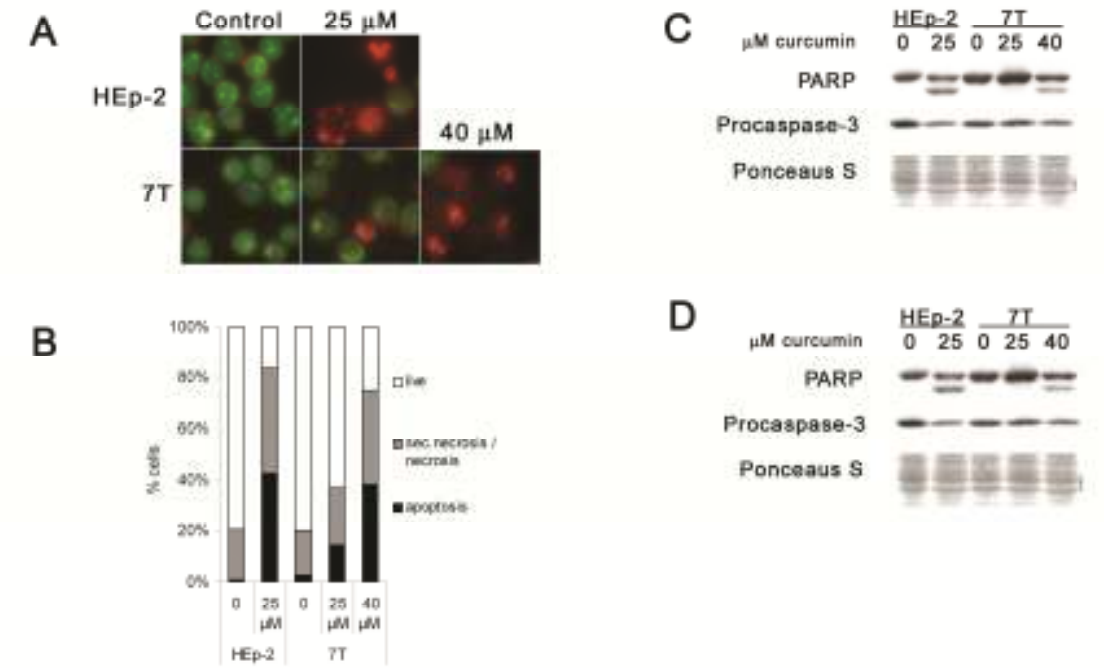

Figure 6. Fatty acid composition of the membranes of HEp-2 and 7T cells. HEp-2 and 7T cells were cultured for 48 h, washed and thereafter fatty acids were extracted from cell lysates and separated by gas chromatography. Proportion of each fatty acid is expressed as a percentage of the total fatty acid content determined. The results are shown as mean values of four experiments (± S.D.).

Supplement Figure S1. The influence of cell density and medium volume on the survival of HEp-2 and 7T cells after the treatment with curcumin. 2.500 HEp-2 cells and 2.500 or 3.500 7T cells were seeded in 67 μl or 180 μl of medium and next day treated with different concentrations of curcumin in final volume of 75 μl or 200 μl, respectively. Both, HEp-2 and 7T cells were incubated for 72 h with curcumin and their survival was determined by MTT assay. Representative of three similar experiments is shown.

Supplement Figure S2. The accumulation of curcumin in HEp-2 and 7T cells after 15 min treatment. The protocol was the same as described for ROS measurement (paragraph in Methods), but without incubation with H_2DCFHDA. During that period of time cells were kept in the cell medium. Thereafter the cells were incubated for 15 min with 25 and 40 μM curcumin, and washed with PBS without Ca^{+2} and Mg^{+2} ions. Intracellular curcumin fluorescence was analyzed on FACSCalibur flow cytometer. The results are shown as mean values of three experiments ± S.D. * $p<0.01$ versus HEp-2; ** $p<0.05$.

Supplement Figure S3. PARP cleavage and Bcl-2 expression during curcumin treatment in HEp-2 and 7T cell line. PARP cleavage and Bcl-2 expression were observed during 3, 6, 12 and 24 h of treatment with equimolar (25 μM) and equitoxic (40 μM) concentrations of curcumin by Western blot analysis. Membrane stained with Ponceau S was used as loading control. Representative of two independent experiments is shown.