

RESEARCH ARTICLE

Curcumin Conjugates Induce Apoptosis Via a Mitochondrion Dependent Pathway in MCF-7 and MDA-MB-231 Cell LinesDurg Vijay Singh^{1,4}, Shikha Agarwal¹, Preeti Singh², Madan Madhav Godbole², Krishna Misra^{3*}**Abstract**

In order to enhance the bioavailability of curcumin its conjugates with piperic acid and glycine were synthesized by esterifying the 4 and 4' phenolic hydroxyls, the sites of metabolic conjugation. Antiproliferative and apoptotic efficacy of synthesized conjugates was investigated in MCF-7 and MDA-MB-231 cell lines. IC₅₀ values of di-O-glycinoyl (CDG) and di-O-piperoyl (CDP) esters of curcumin were found to be comparable with that of curcumin. Both conjugates induced chromatin condensation and apoptotic body formation. CDP exposure to MCF-7 cells induced apoptosis initiating loss of mitochondrial membrane potential ($\Delta\psi_m$) followed by inhibition of translocation of transcription factor NF- κ B and release of Cytochrome-C. Reactive oxygen species (ROS) production was evaluated by fluorescent activated cell sorter. Change in ratio of Bcl2/Bcl1 was observed, suggesting permeabilization of mitochondrial membrane leading to the release of AIF, Smac and other apoptogenic molecules. DNA fragmentation as a hallmark for apoptosis was monitored by TUNEL as well as agarose gel electrophoresis. Thus, it was proven that conjugation does not affect the therapeutic potential of parent molecule *in vitro*, while these could work *in vivo* as prodrugs with enhanced pharmacokinetic profile. Pharmacokinetics of these molecules under *in vivo* conditions is a further scope of this study.

Keywords: Apoptosis - curcumin conjugates - NF- κ B - Bcl₂ - Bax - AIF - immunofluorescence - Western blotting

Asian Pac J Cancer Prev, 14 (10), 5797-5804

Introduction

Curcumin is known for plethora of medicinal properties, however the molecule suffers from poor bioavailability i.e. lower plasma concentration, fast metabolism and high rate of efflux which results in its maximum half life of 90 minutes (Anand et al., 2007). However this molecule is safe upto 12g/day, in oral uptake (Lao et al., 2006). Several conjugates and derivatives of curcumin have been studied for their therapeutic potential. We have synthesized curcumin conjugates with several amino acids including glycine i.e. di-O-glycinoyl curcumin (CDG) (Figure 1B), and with piperic acid, di-O-piperoyl curcumin (CDP) (Figure 1C) and several other bioactive molecules (Mishra et al., 2005a). For glycine there are several transporters in intestinal absorptive epithelia. Conjugation of curcumin with glycine could enhance its cellular uptake by smuggling. Shobha et al., (1998) have reported that piperine enhances the oral bioavailability of curcumin by 2000%. Piperine is an inhibitor of P-glycoprotein (P-gp), hence piperic acid's conjugate of curcumin could enhance the bioavailability of the molecule via modulating the efflux mechanism. Among the synthetic curcumin conjugates CDG and

CDP have shown better antibacterial and antifungal properties than curcumin and its other conjugates (Mishra et al., 2005a). Present study was undertaken to assess the anticancer activity of curcumin conjugates of piperine and glycine vis-a-vis curcumin in MCF-7 and MDA-MB-231 cell lines.

Apoptosis is a self regulatory process, mediated by caspases and some other self controlled mechanisms. Sometimes apoptosis is activated by chemotherapeutic

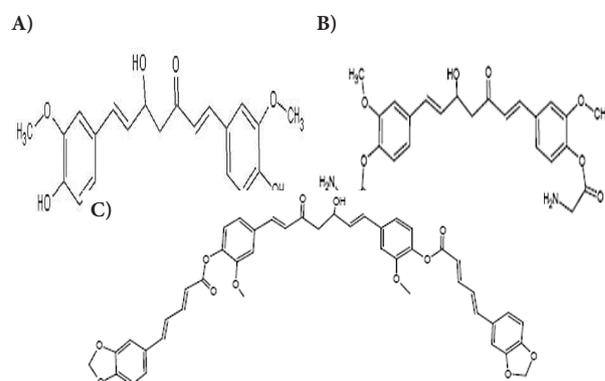


Figure 1. A) Curcumin; B) Di-O-Glycinoyl Curcumin; and C) Di-O-Piperoyl Curcumin

¹Department of Bioinformatics, Indian Institute of Information Technology, Allahabad, ²Department of Endocrinology SGPGIMS, ³Center for Biomedical Magnetic Resonance, SGPGIMS Campus, Lucknow, ⁴Department of Bioinformatics Central University of Bihar, Patna, India *For correspondence: krishnamisra@hotmail.com

drugs. Caspase are cysteine proteases which on activation by external or internal stimuli form central executioner caspases i.e. caspase-3, -6 and -7 (Nunaez et al., 1998). During apoptosis several cellular processes converge on mitochondria (Wang and Youle, 2009). Integrity of mitochondria is important for its energy balance as well as cell survival. Decrease in mitochondrial transmembrane potential and altered cellular redox milieu is cause of early stage of mitochondrial mediated apoptosis (Asa and Roberta, 2008). Intermembrane space of mitochondria has several proteins that can induce apoptosis e.g. Cytochrome C (Kulikov et al., 2012), Smac, (Rudy et al., 2008) HtrA2/Omi (Suzuki et al., 2001). Beside this antiapoptotic proteins Bcl₂/Bcl_{xl} and apoptotic proteins Bid, Bax, Bak and BH3 domain-only proteins play central role (Youle and Strasser 2008; Wang and Youle 2009). This study elucidates that conjugation does not affect the efficacy of parent molecule and induce apoptosis via intrinsic pathways.

Materials and Methods

Synthesis of curcumin conjugates

Synthesis of 4,4'-di-(O-glycinoyl) curcumin (Figure 1B): curcuminoids were extracted from turmeric powder with 95% ethanol. Extract was filtered and ethanol was removed via rotary evaporator. Extracted Curcuminoids were purified by column in order to get pure curcumin as described by Almeida et al. (2005). Curcumin crystallised from aqueous ethanol to yield yellow needles like crystals. 1,7-Bis (4-O-glycinoyl-3-methoxyphenyl)-1,6-heptadiene-3,5-dione was synthesized from sodium salt of curcumin and glycine with slight modification as described by Kumar et al. (2001).

Synthesis of 4,4'-di-(O-piperoyl) curcumin (Figure 1C): piperine was extracted from ground black pepper fruits (100g) in dichloromethane (DCM) (200ml) using Soxhlet (Ikan, 1991). After 2h of reflux DCM was distilled and the residue obtained was purified on column and piperine obtained crystallized from ethanol. Crystallized piperine was refluxed in ethanolic KOH for 4h to obtain piperic acid. Ethanol was distilled and the remaining concentrate was poured into hot water and acidified with 2N HCl to precipitate piperic acid. 4,4'-di-(O-piperoyl) curcumin was synthesized and purified as described by Mishra et al. (2005). Purity of of both conjugates was checked by HPLC and NMR.

Reagents and culture methods: MCF-7 and MDA-MB-231 cells were obtained from National Center for Cell Sciences and grown in F-12 and MEM media respectively, supplemented with 10% FCS, 2% Sodium pyruvate, 1% penicillin, and 1% streptomycin. Cells were grown in T-25/75 flasks to 80% confluency at 37°C also supplemented with 5% CO₂ and humidified air. Cells were routinely trypsinized (0.05% trypsin /EDTA) and subcultured. Curcumin conjugates of CDP and CDG were synthesized in our laboratory.

Cell culture and drug treatment: solutions of curcumin conjugates were prepared in DMSO as stock concentration of 10 mM each and stored at -20°C. For all *in vitro* assay solutions of compounds were diluted serially in the range

of 1 μ m to 100 μ M concentrations. Exponentially growing cells were seeded at the densities that allowed untreated cells to reach nearly confluent state 75-80%. Cells were treated with curcumin conjugates after 24 h of plating in media.

Cell viability assay: cytotoxic effects of curcumin conjugates on MCF-7 and MDA-MB-231 were determined by the trypan blue staining, counting the viable cells on hemocytometer. Briefly cells (1 \times 10⁴ cells/well) were seeded in 12 well plates. After 24 h of plating the cells were exposed to increasing concentrations of curcumin conjugates ranging from 0-100 μ M in serum free media, both attached and non attached cell were collected and resuspended in phosphate buffered saline (PBS). The IC₅₀ values were determined from concentration response curve.

Morphological detection of apoptosis: the cells were fixed for 5min in phosphate buffered saline (PBS) containing 3% paraformaldehyde, washed with PBS, and stained for 10 min in Hoechst33258 (10 μ g/L), mounted in 50% glycerol and stored at -4°C before analysis. Nuclear morphology was evaluated using fluorescence microscopy.

DNA Fragmentation assay: CDP treated and untreated cells (~10⁶) were pelleted and resuspended in hypotonic buffer [25 mM trisHCl (pH7.4), 25 mM EDTA and 0.5% triton X-100] for 30 min on ice. After centrifugation at 10,000 \times g, 0.1mg/ml Rnase-A was added to supernatant for 2h followed by 0.1 mg/ml of proteanase -K for 4hr at 37°C. DNA was extracted using phenol - chloroform and then precipitated with ethanol and sodium acetate. Pelleted DNA was dissolved in tris EDTA (pH 8.0) and electrophoresed on a 1.8% agarose gel in the presence of ethidium bromide (0.5 μ g/ml) and scanned with Geldoc.

TUNEL assay for apoptosis: cells were grown on microscope slides and were treated with CDP based on IC₅₀ value obtained from cell viability assay. The fragmented DNA of apoptotic cells was quantified by TdT mediated dUTP nick labelling (TUNEL) with the apoptotic detection kit. Briefly cells were fixed with 4% paraformaldehyde at 4°C and washed with phosphate buffered saline (PBS) for 30min, to each slide was then added 0.1ml of equilibrium buffer and covered with parafilm for 10min at 37°C. A mixture of 1 μ l TdT (terminal deoxynucleotidil transferase) enzyme, 5 μ l nucleotide mix and 45 μ l equilibrium buffer were prepared in the dark and 50 μ l of the mixture was added on each slide. Next, the slides were incubated for 1 or 2 h at 37°C in a container to protect it from any light source. After that 2 \times SSC were added for 15 min at r.t. to stop the TdT enzymes reaction. After washing with PBS in order to eliminate the unbound fluorescence-dUTP, the slides were immersed in Hoeschst for 15 min in the dark to stain the cells. Slides were dried after rinsing with distilled water and coverslips were later overlaid on the cell area of the slides. This assay specifically detects apoptotic cells when examined through fluorescent microscope.

Measurement of ROS with 2', 7'-dichlorofluorescein diacetate (DCFDA): intracellular ROS generation and mitochondrial membrane potential ($\Delta\psi_m$) were measured using 2', 7'-dichlorofluorescein diacetate (DCF-DA) respectively. Cells were plated in six well plates for 0, 12,

24 and 36 h. Drug dose was given as per the IC_{50} value obtained. DCF-DA ($1\mu M$) was added over 30 minutes to culture medium at $37^{\circ}C$ in dark. Cells were washed with cold PBS, scraped from the plate and resuspended at 1×10^6 cells/ml in PBS containing 10 mM EDTA. The fluorescence intensity of the 2', 7'-dichlorofluorescein formed by the reaction between DCF-DA and the intracellular ROS of more than 10,000 viable cells from each sample was analyzed by flow cytometry at an excitation and emission wavelength of 488 and 525nm, respectively. The experiment was repeated at least three times with similar results. Data were expressed as representative histograms from the independent experiments.

Evaluation of mitochondrial membrane potential ($\Delta\psi_m$) using DiO6/Rhodamine: DiO6 was used to monitor the curcumin conjugates induced mitochondrial transmembrane potential. Briefly, cells were seeded at density 4×10^4 cell/well in six well plates for 0, 12, and 24 h. After drug treatment cells were loaded with probe DiO6 ($50nM/L$) for 30 min at $37^{\circ}C$ before flow cytometric analysis. The supernatant was removed and the cells were harvested and suspended in PBS. Measurement of the retained DiO6 in each sample was counted for 10,000 cells. DiO6 was excited at 488nm and fluorescence was analyzed at 525nm. Mitochondrial membrane potential was also evaluated by fluorescent dye accumulation in mitochondria. Briefly cells were grown into six well plates over coverslips and treated with CDP for 0, 12 and 24 h. Media was removed and washed with PBS and probed with rhodamine in serum free media for 20min and incubated at $37^{\circ}C$. Cells were washed twice with PBS and fixed with 4% paraformaldehyde and mitochondrial membrane potential inspected by fluorescence microscopy at $40\times$.

Immunofluorescence: cells were grown on coverslips as described above, treated with CDP, fixed in 4% paraformaldehyde for 30 min at $4^{\circ}C$. Cells were washed with PBS for 5 min and permeabilized with pre-chilled 0.2% triton-X-100/PBS. Non specific sites were blocked by incubating with $100\mu l$ of 2% bovine serum albumin in PBS at $37^{\circ}C$ for 15 min. A mouse monoclonal antibody (primary antibody) against N-terminal of Bax YTH-6A7 (trivigen, Gaithersberg, MD) and goat polyclonal anti-AIF E-1 (Santa Cruz Biotechnology, SANTA Cruz, CA) were used. Before labeling with anti-Bax, cells were permeabilized for 5 min with 0.0125% CHAPS/PBS to prevent artificial activation of Bax (Antonsson et al., 2000). Cells were then washed with 2% bovine serum albumin/PBS for 10 min at r.t. before incubating with secondary antibodies. Secondary antibodies: FITC conjugated immunoglobulins (DAKO Denmark) were incubated for 1h coverslips were rinsed with 2% BSA for 10 min and counterstained with Hoechst-33258 ($10\mu g/ml$). Hoechst staining of the nuclei was used to visualize the apoptotic nuclei. Cells were photographed under conventional UV-fluorescence microscope.

Cytosolic and subcellular protein preparation: cells were incubated for 12, 24 and 48h time periods at the concentration to induce apoptosis. Cells were harvested without trypsinisation, centrifuged at $200\times g$, washed

twice with PBS (pH 7.2) and were then pelleted again at $200\times g$. Cells were resuspended in 5 ml of ice-cold buffer (20 mM HEPES, pH7.5, 1.5 mM $MgCl_2$, 250mM sucrose, 1mM EDTA, 1mM EGTA, DTT and protease inhibitors cocktails) and kept on ice for 5 min to break open cells and release nuclei using a pre-chilled Dounce homogenizer. Homogenized cell preparation was centrifuged at $800\times g$ for 10 min at $4^{\circ}C$ to pellet nuclei and supernatant as a cytoplasmic fraction (Hegde et al., 2002). Again supernatant (cytoplasmic fraction) was centrifuged at $10000\times g$ to pellet mitochondria and supernatant was gently separated as cytosolic fraction. In cytosolic fraction RIPA buffer (50 mM Tris, pH 7.5, 50 mM NaCl, 1% triton X-100, 0.5% SDS, and protease inhibitor cocktail along with (PMSF, 0.1 mM, sodium orthovanadate 1mM, and aprotinin and leupeptin $2\mu g/ml$) were mixed well and then centrifuged at $10000\times g$ for 10 min at $4^{\circ}C$ to pellet out any solids, supernatant as cytosolic protein preparation stored at $-20^{\circ}C$. Mitochondrial fraction was subjected to freeze-thaw in RIPA buffer and centrifuged at $10000\times g$ to remove any solid pellets, supernatant was stored at $-20^{\circ}C$ as a crude mitochondrial protein preparation. Resuspended nuclear pellet in (0.88 mM sucrose, 5 mM $MgCl_2$, and protease inhibitors) centrifuged at $2800\times g$ for 10 min at $4^{\circ}C$, resulted into a cleaner nuclear pellet. Nuclear pellet was mixed with RIPA buffer and then frozen and thawed to ensure release of as many nuclear proteins as possible. Lysate was centrifuged at $2800g$, for 10 min at $4^{\circ}C$ to pellet any solids; supernatant was stored $-20^{\circ}C$ as nuclear protein fraction.

Western blot analysis: the protein concentration was measured using Bradford dye absorption spectra, taken at 550nm. After boiling for 10min in the presence of 2-mercaptoethanol, samples containing cell lysate protein were separated on a 12 or 15% sodium dodecyl sulphate-polyacrylamide (SDS) gel and then transferred onto equilibrated nitrocellulose membranes. After skimmed milk blocking, the membranes were incubated with primary antibodies individually with Cytochrome-C, caspase-7, Bax, NF-kB (P65unit), Bcl2, BclXL, AIF, Smac and P53 (1:1000 dilution). The bound antibodies were detected with horsereddish peroxidase-labelled sheep anti-mouse IgG or hosereddish peroxidase-labelled ant-rabbit IgG. The immunoblots were then developed with automated developer. Bax NF-kB and AIF nuclear extract were prepared separately on treatment of CDP for 16, 32 and 48h. (Witcher et al., 2003). Nuclei were pelleted by lysing in hypertonic buffer. Protein concentration was determined using Bradford reagent. Equal amount of protein ($50\mu g$) was loaded in each lane and resolved on 12% gel.

Statistical analysis

All the results are expressed as mean \pm SE (standard error) from one of the three similar experiments performed in triplicate.

Results

The two conjugates of curcumin viz. 4,4'-O-diglycinoyl and 4,4'-O-dipiperoyl esters were synthesized

in our laboratory as described by Kumar et al. (2001) and Mishra et al. (2005). Both curcumin diesters were synthesized from sodium salt of curcumin; by a slightly modified reaction scheme as proposed by Kumar et al. (2001) and Mishra et al. (2005) in order to increase the yield.

Cytotoxicity of curcumin conjugates in MCF-7 and MDA-MB-231 breast cancer cell lines: Cell viability was measured using the trypan blue dye exclusion methods. Drug response curves were drawn on treatment with increasing concentration of curcumin conjugates. Effect of CDG, and CDP on MCF-7 and MDA-MB-231 was comparable to curcumin. CDP shows IC₅₀ 33.6μM and 44.2μM in MCF-7 and MDAMB cell lines respectively. While CDG shows IC₅₀ as 51μM and 55.8μM in MCF-7 and MDA-MB-231 cell lines respectively (Figure 2). The cell number of both cell line decreases in 96 h and less than 25% cells survives. CDG and CDP both conjugates have shown time dependent effect also (Figure 3A and 3B). Cell morphology on treatment of curcumin conjugates was examined at their IC₅₀ value. Cells treated with conjugates gets rounded up and detached from culture plates. Curcumin has shown the IC₅₀ 30μM and 45μM in MCF-7 and MDAMB-231 cell lines respectively in our lab conditions. Curcumin was not tested further since pathways and mechanism of cell death for curcumin is very well established.

Effects on cellular and nuclear morphology: in order to check whether at IC₅₀ value conjugates induced cell death,

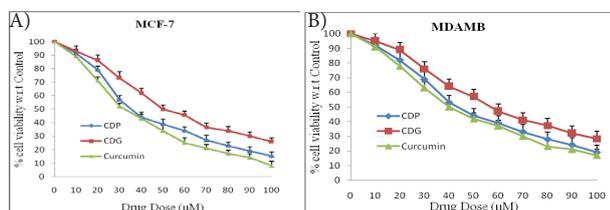


Figure 2. Dose Dependent Effect of Curcumin and Curcumin Conjugates in A) MCF-7; and B) MDA-MB-231 Cell Lines. Cells were plated at the density of 4×10⁴ cells/ml in a 12-well plate and were treated with concentrations of (0 to 100μM) curcumin conjugates for 72 h. After 72 h of treatment all cell floating and non floating were subjected to cell % viability assay. Cell viability was analyzed by the trypan blue dye exclusion method. The negatively stained cells were counted in a hemocytometer. The percent of viable cells is expressed as mean of values obtained in three experiments

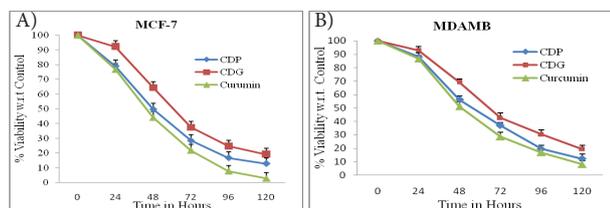


Figure 3. Time Dependent Effect of Curcumin and Curcumin Conjugates in A) MCF-7; and B) MDA-MB-231 Cell Lines. Cells were plated at the density of 4×10⁴ cells/ml in 12-well plates and were treated with IC₅₀ concentrations of curcumin conjugates for 120 h. Cell viability was analyzed using the trypan blue dye exclusion method. The negatively stained cells were counted in a hemocytometer. The percent of viable cells is expressed as mean of values obtained in three experiments

the cells were stained with fluorescent DNA binding dye (Hoechst-33258). These cells were qualitatively analyzed on treatment of CDG and CDP. Microscopic observation of cellular nucleus of MCF-7 cells has shown typical features of apoptosis i.e. nuclear fragmentation, blebbing, chromatin condensation and small apoptotic bodies that are peculiar feature of apoptotic cells (Ziegler and Groscurth, 2004). While nuclei of MDMB cell lines have shown blebbing, bilobed, crescent like structures but not nuclear fragmentation and chromatin condensation as shown in MCF-7 cells (Figure 4).

DNA fragmentation: to further confirm CDP induced apoptosis in MCF-7 cell line DNA laddering pattern was analyzed at IC₅₀ concentration on 24, 48 and 72 h of treatment. MCF-7 cells have shown internucleosomal DNA laddering Pattern (Saraste and Pulkki, 2000). End effect of CDP MCF-7 cells were analyzed for TUNEL assay, a technique which used to find out end result in

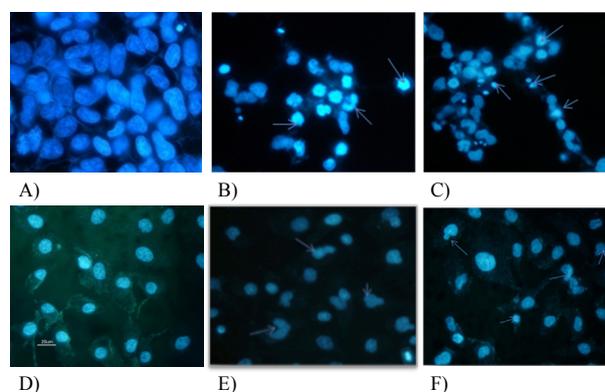


Figure 4. Curcumin Conjugate Induced Change in Nuclear Morphology of MCF-7 and MDA-MB-231 Cells. A) Is a control MCF-7 cell lines; B) and C) Are CDG and CDP treated cells respectively; D) Is a control MDA-MB-231 cell lines; E) and F) are treated with CDG and CDP respectively. Cells were plated at the density of 4×10⁴ cells/ml in 6-well plate over coverslips and were treated with IC₅₀ concentrations of curcumin conjugates for 48 h. nuclear morphology was observed by Hoechst-33258 staining (40× objective). Nuclei exhibited condensation, margination, and fragmentation after 72 h of treatment while nuclear fragmentations were not seen in MDA-MB-231 cells

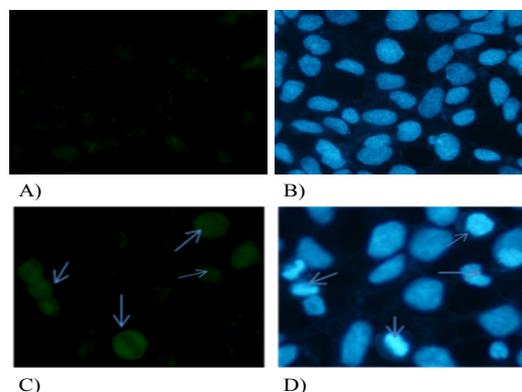


Figure 5. TUNEL Assay for Apoptosis. Cells were grown on coverslips and treated with the IC₅₀ concentrations of CDP for 36h and apoptosis was analyzed by TUNEL assay. A) Control for TUNEL assay; B) Nuclei of control cells stained with Hoechst; C) Green fluorescence of cells showing TUNEL positive; and D) Nuclei of treated cells stained with Hoechst

programmed cell death (Lesauskaite and Ivanoviene, 2002). TUNEL assay has shown number of apoptotic cells on 36 h of treatment (Figure 5).

Curcumin conjugates induced apoptosis is associated with ROS generation and loss of $\Delta\psi_m$ in MCF-7 cell lines: Several characteristics of early apoptotic events involve enhanced ROS production and Loss of $\Delta\psi_m$ (Hail and Lotan, 2000). The study has been conducted to determine the involvement of ROS of the mitochondria-mediated pathway of apoptosis on CDP treatment measured by flowcytometry. ROS production causes loss of mitochondrial membrane potential. To determine the association of ROS/hydroperoxide generation with apoptosis in MCF-7 cell line, the oxidation of DCF was monitored by flowcytometry which has shown more than two fold increase (Figure 6). Decrease in mitochondrial membrane potential $\Delta\psi_m$ mediated by the opening of mitochondrial multicomplex pore in a process termed as permeability transition. Decrease in mitochondrial membrane potential was determined by FACS. Treatment of CDP for 24 h has shown six fold increase in fluorescence (Figure 6). This process has suggested necessarily the release of apoptogenic molecules in the cytosols (Zamzami et al., 1996; Susin et al., 1997) mitochondrial membrane integrity was also measured by rhodamine

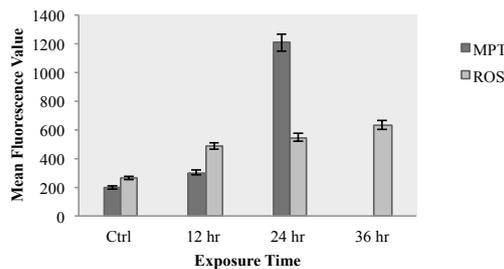


Figure 6. Light Gray Color Bar is Showing Time Dependent (12, 24 and 36 hrs) Measurement of CDP Induced Hydro-Peroxide Generation in MCF-7 Cells, Measured by Flowcytometry using DCF-DA as a Measure of ROS. Dark gray color bar is showing time dependent change in mitochondrial membrane potential ($\Delta\psi_m$) for 12 and 24 hr by flow cytometry with DiO6. Increase in fluorescence intensity shows the loss of mitochondrial membrane potential (MPT). All exp was performed at control/ IC_{50} and data represents the means of three individual experiments with error bars

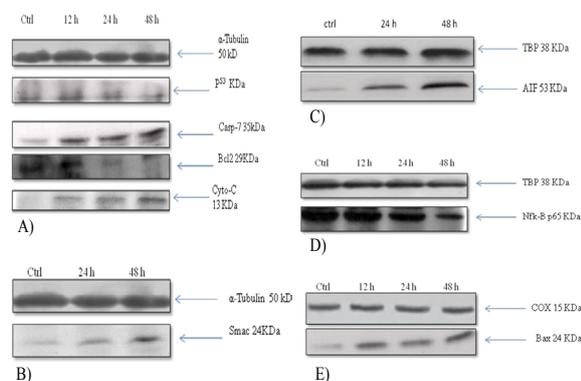


Figure 7. MCF-7 Cells were Treated with IC_{50} Concentration of CDP for 16, 32, and 48 h or Left Untreated. The protein extracts (50 μ g) were separated on a 12% SDS-polyacrylamide gel and immunoblotted

accumulation in mitochondria. On 24 hr treatment of CDP, fluorescent microscopical observation has shown diffused rhodamine fluorescence in cytosol (Hsu et al., 2007).

Role of members of Bcl₂ family proteins in mitochondrial apoptosis: Bcl₂, Bcl_{xl} and Bax are the major molecules which play significant role in mitochondrial pathway of apoptosis. Curcumin conjugate CDP induction altered expression of these members of proteins and were monitored by Western blots. Bcl₂'s expression (Figure 7A) decreases in time dependent manner while change in expression of Bcl_{xl} was not observable. Bax is an apoptosis inducing proteins which is over expressed (Figure 7E). Bax gene, is an apoptosis promoter, regulates the release of Cytochrome C from mitochondria (Shimizu et al., 1999) and its forced expression is known to lead to a cascade of activation of caspases and to programmed cell death (Rosse et al., 1998; Kagawa et al., 2000). However it is controversial whether caspases are required for Bax induced apoptosis? Both caspase-dependent cell death (kitanaka et al., 1997) and caspase independent cell death (Xiang et al., 1996) have been reported. Bax is known to be transcriptional target of P53 (Miyashita and Reed, 1995). However Western blot analysis for P53 on CDP treatment has not shown any change in its expression level.

Release of Cytochrome C during downstream process of loss of mitochondrial membrane potential (MMP): loss of MMP further causes several changes in mitochondrial environment to initiates apoptosis. Cytochrome C is normally physically associated with the inner mitochondrial membrane facing intermembrane space. CDP treatment has shown release of Cytochrome-C into cytosol (Figure 7A). Release of Cytochrome C into cytosol initiates downstream processes i.e. caspase activation via a complex of caspase-9 and Apaf-1. MCF-7 has shown apoptosis while it lacks caspase-3. Despite this, MCF-7 cells undergo morphological apoptosis after treatment with a variety of agents and conditions (Oberhammer et al., 1993; Eck-Enriquez et al., 2000; liang, 2001).

SMAC/Dialbo: loss of mitochondrial membrane potential induces release of mitochondrial proapoptotic protein Smac, Smac/Dialbo binds to XIAP thereby antagonizing XIAP interaction with caspase 9 and promoting the activity of caspase 9, followed by caspase 3 and apoptosis. CDP causes release of Smac apoptogenic molecule shown by Western blot (Figure 7B).

Release of AIF and nuclear translocation: CDP induced release of AIF (Figure 7C) and nuclear translocation was observed by Western blot and immunofluorescence. MCF-7 cells lack the functional caspase-3 which is a major executioner caspase protease. It seems that in absence of caspase-3, AIF plays a major role in CDP induced apoptosis. Since it is well known that apoptosis is governed by myriad of cell signaling molecules and proteins, it is difficult to isolate each one due to overlap and shared signaling pathways. It has been shown that two or more than two pathways can exist simultaneously in the same cell. Cell may switch back and forth between concomitantly operating death pathways and dominant cell death features is decided by the comparative speed of other death pathways (Chi et al., 1999), although attributes of several death pathways can be seen, only the fastest and

most striking death pathway is generally observed (Bursch et al., 2001).

Role of NF- κ B in cell survival and apoptosis: on activation, I κ B α subunit of NF- κ B undergoes phosphorylation and degradation to expose nuclear localization signals on the p50-p65 heterodimer, leading to its nuclear translocation. In the nucleus it binds to specific consensus sequences in the DNA (5'-GGGACTTTC-3') called κ B binding site. Some genes like Cyclin D1, apoptosis suppressor proteins such as Bcl₂, Bcl_{xl}, VEGF, MMPs are the targets for nuclear factor - κ B (NF- κ B) and critical to the establishment of early and late carcinogenesis (Aggarwal and Shishodia, 2004; Hussain et al., 2008). Curcumin suppresses activation of NF- κ B as reported by several authors. Therefore, agents that can suppress NF- κ B activation have potential to prevent or delay the onset of or treat NF- κ B linked cancers (Yu et al., 2011). We report the decrease in expression of NF- κ B in nuclear extract of MCF-7 cell line on treatment with CDP (Figure 7D). Inhibition of NF- κ B translocation on treatment of CDP was also shown by immunofluorescence further support our findings.

Discussion

Curcumin is pharmacologically safe upto 12g/day but due to poor oral bioavailability and high rate of metabolic detoxification it does not reach upto optimal plasma concentration required for therapeutic potency. Our long term approach is to enhance oral bioavailability and plasma retention time by blocking metabolic sites of the molecule. Most hydrophobic drug molecules like curcumin are effluxed through MDR (multidrug resistance) protein P-gp (P-glycoprotein). Pgp is highly expressed in most of the biological gates where it gets into intestinal epithelia of kidney, liver and blood brain barriers; Piperine enhances the oral bioavailability of curcumin Shoba et al. (1998) and also computationally shown by (Singh et al, 2013). Esterification of curcumin with piperic acid and amino acids like glycine block its phenolic functions which are the metabolic sites for sulfatation and glucuronidation. Thus the metabolic degradation rate is delayed. Piperine should overcome the efflux problem and amino acids could smuggle curcumin inside cells via natural transporters. Glycine is an essential building block of all living cells, therefore can drag (smuggle) curcumin molecule inside cells enhancing bioavailability. Carrying forth the above hypotheses these molecules were covalently linked i.e. curcumin and piperine were extracted from their natural resources and their conjugates were synthesized. Whether antitumor properties of these covalently linked molecules were retained or compromised; in vitro testing were performed in MCF-7 and MDA-MB-231 cell lines.

In the present work, we elucidated the mechanism of cell death induced by curcumin conjugates i.e. CDP and CDG in MCF-7 and MDA-MB-231 breast cancer cell line. In both the cell lines the IC₅₀ of CDG and CDP were comparable with curcumin. Both conjugates have shown time and dose dependent response (Figure 2). In order to further investigate whether the cell death is due to necrosis or a programmed cell death in MCF-7 and MDA-MB-231

were stained by Hoechst, a fluorescent dye to find out change in nuclear morphology (Figure 4). Fluorescently stained cells have shown apoptotic features in both cell lines. Finally cells were also examined by TUNEL assay to confirm that cell death occurs via apoptosis. MDA-MB-231 cells have shown cell death at a little higher concentration than MCF-7. DNA laddering along with TUNEL positive cells characteristics of apoptosis was observed on CDP treatment.

Cell system is evolved in such a fashion so that it encounters against any external stimuli and if fails to survive it shifts to death via necrosis or programmed cell death (apoptosis). CDP treatment in MCF-7 cell line has shown alteration in ratio of Bcl₂ and Bax (Figure 7A and 7E). Curcumin also has shown alteration in Bcl₂ and Bax ratio in several cell lines. Bcl₂ and Bcl_{xl} both are antiapoptotic proteins which extend cell survival against many anticancer stimuli. Another family of proteins i.e. Bax and Bak induce apoptosis in cells. Hence the ratio antiapoptotic vs apoptotic protein is important to lead the way of mitochondrial dependent apoptosis (Chen et al., 2010; Kunwar et al., 2012). Bax translocates to mitochondria and leads to membrane permeabilization. In BH3 domain only members of Bcl₂ family proteins induce conformational changes and activate Bax (Bleicken et al., 2010; Westphal et al., 2011). Moreover Bax and Bak oligomerization-mediated membrane perforation occurs upstream to release AIF and endonuclease G (Asa and Roberta, 2008). Bax is known to form large lipidic pores through which high molecular weight molecules can pass (Bleicken et al., 2010). Rashmi et al. (2005) have shown role of Bax in curcumin-induced apoptosis using isogenic human colon cancer cells (HCT 116). The results from their study suggest that Bax deficiency almost completely prevented the release of Cytochrome C, AIF and Smac with subsequent inhibition of caspases 3, 8 and 9. The reintroduction of Bax causes overexpression of Smac or AIF, or the downregulation of Bcl_{xl}.

The present work has confirmed that CDP treatment induced ROS production similar to curcumin and induces apoptosis via mitochondrial pathway. Therefore CDP is a major source for ROS production (Anto et al., 2002; Uddin et al., 2005). It is possible that curcumin activates mitochondrial enzymes that lead to production of ROS. There are several reports which suggest that curcumin works as pro-oxidant (Anto et al., 2002; Atsumi et al., 2005; Strasser et al., 2005) and antioxidant (Mishra et al., 2005b; Barzegar and Moosavi, 2011). Moreover, it is reported that curcumin quenches ROS at lower levels and induces the same at higher concentration. It is well established that curcumin interact with thioredoxin reductase irreversibly (Fang et al., 2005) also has been confirmed by computational methods (Singh and Misra, 2009). Curcumin alters the activity of NADPH oxidase leading to production of ROS (Fang et al., 2005). Thioredoxin reductase catalyzes NADPH dependent reduction of thioredoxin which plays an essential role in substrate reduction defense against oxidative stress and redox regulation. Further we investigated the release of Cytochrome C in cytosolic fraction of MCF-7 cells (Figure 7A). Release of Cytochrome C is a hallmark for

caspase dependent cell death. However, MCF-7 cells lack caspase-3 which is the main executioner caspase of cell death. Caspase-7 is similar to caspase-3 which works on most of the substrates of caspase-3. In absence of caspase-3, caspase-7 is known to takeover the function of caspase-3. Walsh et al. (2008) have shown that out of twenty twelve substrates preferentially processed by caspase-3, only one (cochaperone p23) was more susceptible to proteolytic processing by caspase-7. Yang et al. (2001) have shown reconstitution of caspase-3 which sensitizes MCF-7 breast cancer cells to doxorubicin- and etoposide-induced apoptosis while MCF-7 is resistant to both drugs while CDP is sensitive to MCF-7 in absence of functional caspase-3.

Loss of mitochondrial membrane potential induces release of Cytochrome C, Smac/DiALBO, Omi/H2rA2, AIF and Endo G protein. Release of Smac, a mitochondrial proapoptotic protein is followed with its binding to XIAP, by which it antagonizes XIAP interaction with caspase 9, thereby promoting the activity of caspase 9, followed by caspase 3 and apoptosis. Fandy et al. (2008) have shown that Smac/DIABLO gene or cell permeable Smac/DIABLO peptide enhances the apoptosis-inducing potential of chemotherapeutic drugs and irradiation, and sensitizes TRAIL-resistant breast cancer cells to apoptosis. CDP induced cell death has shown increased expression of AIF in nuclear extract in time dependent manner (Figure 7C). Immunofluorescence of AIF translocation to nucleus further confirmed AIF induced cell death in MCF-7 cell line. The lack of functional caspase-3 MCF-7 might be the cause for shift of pathway i.e. induction of AIF mediated cell death. NF- κ B is another important macromolecule required for cell survival which normally resides in cytosol and upon activation it gets translocated to nucleus induces expression of several transcription factors which are required for cell survival and growth. CDP inhibits translocation of this molecule to nucleus therefore inhibits the activity of the molecule. Western blot of NF- κ B from nuclear extract has shown decrease in expression which is also supported by immunofluorescence.

In conclusion, considering all the above experimental facts it is concluded that CDP induced apoptosis involves activation of Bax, redistribution of AIF from mitochondria to nucleus, release of mitochondrial apoptogenic molecule Cytochrome C and Smac as well as depletion of Bcl₂ and inhibition of Nf- κ B translocation. Treatment with CDP clearly suggests the role of mitochondrial mediated cell death in MCF-7 cell line. However, along with *in vitro* studies, *in vivo* study is must in order to understand the therapeutic potential of curcumin conjugates in breast neoplasia with their enhanced pharmacokinetics as prodrugs. Based on *in vitro* data showing comparable proapoptotic activity of the conjugates to that of curcumin, if pharmacokinetic (PK) data reveals an x-fold increase in systemically achievable levels, then these compounds could have value in delivering pharmacologic doses of drug to target tissues.

Acknowledgements

One of the authors (Durg Vijay Singh) wishes to

express his gratefulness to CSIR, Govt. of India for providing senior research fellowship.

References

- Aggarwal BB, Shishodia S (2004). Suppression of the nuclear factor-kappaB activation pathway by spice-derived phytochemicals: reasoning for seasoning. *Ann NY Acad Sci*, **1030**, 434-41.
- Almeida LP, Cherubino APF Alves RJ, Dufosse L, Gloria MBA (2005). Separation and determination of the physico-chemical characteristics of curcumin, demethoxycurcumin and bisdemethoxycurcumin. *Food Res Int*, **38**, 1039-44.
- Anand P, Kunnumakkara AB, Newman RA, Aggarwal BB (2007). Bioavailability of curcumin: problem and promises. *Mol Pharm*, **4**, 807-18.
- Anto RJ, Mukhopadhyay A, Denning K, Aggarwal BB (2002). Curcumin (diferuloylmethane) induces apoptosis through activation of caspase-8, BID cleavage and cytochrome c release: its suppression by ectopic expression of Bcl₂ and Bcl_{xl}. *Carcinogenesis*, **23**, 143-50.
- Antonsson B, Montessuit S, Lauper S, Eskes R, Martinou JC (2000). Bax oligomerization is required for channel-forming activity in liposomes and to trigger cytochrome c release from mitochondria. *Biochemistry*, **345**, 271-8.
- Asa BG, Roberta AG (2008). Heart mitochondria: gates of life and death. *Cardiovasc Res*, **77**, 334-43.
- Atsumi T, Fujisawa S, Tonosaki K (2005). Relationship between intracellular ROS production and membrane mobility in curcumin and tetrahydrocurcumin-treated human gingival fibroblasts and human submandibular gland carcinoma cells. *Oral Dis*, **11**, 236-42.
- Barzegar A, Moosavi-Movahedi AA (2011). Intracellular ROS protection efficiency and free radical-scavenging activity of curcumin. *PLoS One*, **6**, 26012.
- Bleicken S, Classen M, Padmavathi PV, et al (2010). Molecular details of Bax activation, oligomerization, and membrane insertion. *J Biol Chem*, **285**, 6636-47.
- Bursch W (2001). The autophagosomal-lysosomal compartment in programmed cell death. *Cell Death Differ*, **8**, 569-81.
- Chen QY, Lu GH, Wu YQ, et al (2010). Curcumin induces mitochondria pathway mediated cell apoptosis in A549 lung adenocarcinoma cells. *Oncol Rep*, **23**, 1285-92.
- Chi S, Kitanaka C, Noguchi K (1999). Oncogenic ras triggers cell suicide through the activation of a caspase-independent cell death program in human cancer cells. *Oncogene*, **18**, 2281-90.
- Eck-Enriquez K, Kiefer TL, Spriggs LL, Hill SM (2000). Pathways through which a regimen of melatonin and retinoic acid induces apoptosis in MCF-7 human breast cancer cells. *Breast Cancer Res Treat*, **61**, 229-39.
- Fandy TE, Shankar S, Srivastava RK (2008). Smac/DIABLO enhances the therapeutic potential of chemotherapeutic drugs and irradiation, and sensitizes TRAIL-resistant breast cancer cells. *Mol Cancer*, **7**, 60.
- Fang J, Lu J, Holmgren A (2005). Thioredoxin reductase is irreversibly modified by curcumin: a novel molecular mechanism for its anticancer activity. *J Biol Chem*, **280**, 25284-90.
- Hail NJr, Lotan R (2000). Mitochondrial permeability transition is a central coordinating event in N-(4-hydroxyphenyl) retinamide-induced apoptosis. *Cancer Epidemiol Biomarkers Prev*, **9**, 1293-301.
- Hegde R, Srinivasula SM, Zhang Z, et al (2002). Identification of Omi/HtrA2 as a mitochondrial apoptotic serine protease that disrupts inhibitor of apoptosis protein-caspase interaction. *J Biol Chem*, **277**, 432-8.

- Hsu YC, Weng HC, Shiurulin, Chien Y (2007) Curcuminoids—cellular uptake by human primary colon cancer cells As quantitated by a sensitive HPLC assay and its relation with the inhibition of proliferation and apoptosis. *J Agric Food Chem*, **55**, 8213-22.
- Hussain AR, Ahmed M, Al-Jomah NA, et al (2008). Curcumin suppresses constitutive activation of nuclear factor-kappa B and requires functional Bax to induce apoptosis in Burkitt's lymphoma cell lines. *Mol Cancer Ther*, **7**, 3318-29.
- Ikan R (1991). Natural Products, A Laboratory Guide, 2nd ed.; Academic Press: New York.
- Kagawa S, Pearson SA, Ji L, et al (2000). Binary adenoviral vector system for expressing high levels of the proapoptotic gene bax. *Gene Ther*, **7**, 75-9.
- Kitanaka C, Namiki T, Noguchi K, et al (1997). Caspase-dependent apoptosis of COS-7 cells induced by bax overexpression: differential effects of Bcl₂ and Bcl_{xl} on bax-induced caspase activation and apoptosis. *Oncogene*, **15**, 1763-72.
- Kulikov AV, Shilov ES, Mufazalov IA, et al (2012). Cytochrome c: the Achilles' heel in apoptosis. *Cell Mol Life Sci*, **69**, 1787-97.
- Kumar S, Narain U, Tripathi S, Misra K (2001). Syntheses of curcumin bioconjugates and study of their antibacterial activities against β -Lactamase-Producing Microorganisms. *Bioconjugate Chem*, **12**, 464-9.
- Kunwar A, Jayakumar S, Srivastava AK, Priyadarsini KI (2012). Dimethoxycurcumin-induced cell death in human breast carcinoma MCF7 cells: evidence for pro-oxidant activity, mitochondrial dysfunction, and apoptosis. *Arch Toxicol*, **86**, 603-14.
- Lao CD, Ruffin MT, Normolle D, et al (2006). Dose escalation of curcuminoids formulation. *BMC Complement Altern Med*, **6**, 10.
- Lesauskaite V, Ivanoviene L (2002). Programmed cell death: molecular mechanisms and detection. *Medicina Kaunas*, **38**, 869-75.
- Liang Y, Yan C, Schor NF (2001). Apoptosis in the absence of caspase-3. *Oncogene*, **20**, 6570-8.
- Mishra S, Kapoor N, Mubarak Ali A, et al (2005b). Differential apoptotic and redox regulatory activities of curcumin and its derivatives. *Free Radic Biol Med*, **38**, 1353-60.
- Mishra S, Narian U, Mishra R, Misra K (2005a). Design development and synthesis of mixed bioconjugates of piperic acids-glycine, curcumin-glycine, /alanine and curcumin-glycine –piperic acids and their antibacterial and antifungal properties. *Bioorg Med Chem*, **13**, 1477-86.
- Miyashita T, Reed JC (1995). Tumor suppressor p53 is a direct transcriptional activator of the human bax gene. *Cell*, **80**, 293-9.
- Nunaez G, Benedict MA, Yuanming H, Inohara N (1998). Caspases: the proteases of the apoptotic pathway. *Oncogene*, **17**, 3237-45.
- Oberhammer F, Wilson JW, Dive C, et al (1993). Apoptotic death in epithelial cells: cleavage of DNA to 300 and/or 50 kb fragments prior to or in the absence of internucleosomal fragmentation. *EMBO J*, **12**, 3679-84.
- Rashmi R, Kumar S, Karunagaran D (2005). Human colon cancer cells lacking bax resist curcumin-induced apoptosis and bax requirement is dispensable with ectopic expression of smac or downregulation of Bcl_{xl}. *Carcinogenesis*, **26**, 713-23.
- Rosse T, Olivier R, Monney L, et al (1998). Bcl₂ prolongs cell survival after Bax-induced release of cytochrome C. *Nature*, **391**, 496-9.
- Rudy A, Anton NL, Barth N, et al (2008). Role of smac in cephalostatin-induced cell death. *Cell Death Differ*, **15**, 1930-40.
- Saraste A, Pulkki K (2000). Morphologic and biochemical hallmarks of apoptosis. *Cardiovasc Res*, **45**, 528-37.
- Shimizu S, Narita M, Tsujimoto Y (1999). Bcl₂ family proteins regulate the release of apoptogenic cytochrome c by the mitochondrial channel VDAC. *Nature*, **399**, 483-7.
- Shoba G, Joy D, Joseph T, et al (1998). Influence of piperine on pharmacokinetics of curcumin in animals and human volunteers. *Planta Medica*, **64**, 353-6.
- Singh DV, Godbole MM, Misra K (2013). A plausible explanation for enhanced bioavailability of P-gp substrates in presence of piperine: simulation for next generation of P-gp inhibitors. *J Mol Mod*, **19**, 227-38.
- Singh DV, Misra K (2009). Curcuminoids as inhibitors of thioredoxin reductase: a receptor based pharmacophore study with distance mapping of the active site. *Bioinformation*, **4**, 187-92.
- Strasser EM, Wessner B, Manhart N, Roth E (2005). The relationship between the anti-inflammatory effects of curcumin and cellular glutathione content in myelomonocytic cells. *Biochem Pharmacol*, **70**, 552-9.
- Susin SA, Zamzami N, Castedo M, et al (1997). The central executioner of apoptosis: multiple connections between protease activation and mitochondria in Fas/APO-1/CD95- and ceramide-induced apoptosis. *J Exp Med*, **186**, 25-37.
- Suzuki Y, Imai Y, Nakayama H, et al (2001). A serine protease, HtrA2, is released from the mitochondria and interacts with XIAP, inducing cell death. *Mol Cell*, **8**, 613-21.
- Uddin S, Hussain AR, Manogaran PS, et al (2005). Curcumin suppresses growth and induces apoptosis in primary effusion lymphoma. *Oncogene*, **24**, 7022-30.
- Walsh JG, Cullen SP, Sheridan C, et al (2008). Executioner caspase-3 and caspase-7 are functionally distinct proteases. *Proc Natl Acad Sci*, **105**, 12815-9.
- Wang C, Youle RJ (2009). The role of mitochondria in apoptosis. *Annu Rev Genet*, **43**, 95-118.
- Westphal D, Dewson G, Czabotar PE, Kluck RM (2011). Molecular biology of Bax and Bak activation and action. *Biochim Biophys Acta*, **1813**, 521-31.
- Witcher M, Ross DT, Rousseau C, Deluca L, Miller WH Jr (2003). Synergy between all-trans retinoic acid and tumor necrosis factor pathways in acute leukemia cells. *Blood*, **102**, 237-45.
- Xiang J, Chao DT, Korsmeyer SJ (1996). BAX-induced cell death may not require interleukin 1 beta-converting enzyme-like proteases. *Proc Natl Acad Sci*, **93**, 14559-63.
- Yang XH, Sladek TL, Liu X, et al (2001). Reconstitution of caspase-3 sensitizes MCF-7 breast cancer cells to doxorubicin- and etoposide-induced apoptosis. *Cancer Res*, **61**, 348-54.
- Youle RJ, Strasser A (2008). The Bcl₂ protein family: opposing activities that mediate cell death. *Nat Rev Mol Cell Biol*, **9**, 47-59.
- Yu LL, Wu JG, Dai N, Yu HG, Si JM (2011). Curcumin reverses chemoresistance of human gastric cancer cells by downregulating the NF- κ B transcription factor. *Oncol Rep*, **26**, 1197-203.
- Zamzami N, Susin SA, Marchetti P, et al (1996). Mitochondrial control of nuclear apoptosis. *J Exp Med*, **18**, 1533-44.
- Ziegler U, Groscurth P (2004). Morphological features of cell death. *News Physiol Sci*, **19**, 124-8.