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Augmentation of the Cytotoxic Effects of Zinc Oxide Nanoparticles by MTCP Conjugation: Non-canonical Apoptosis and Autophagy Induction in Human Adenocarcinoma Breast Cancer Cell Lines

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Abstract

Zinc oxide nanoparticles are very toxic, but their agglomeration reduces their lethal cytotoxic effects. Here we tested the hypothesis that conjugation of ZnO nanoparticles via Meso-Tetra (4-Carboxyphenyl) Porphyrin (MTCP) could provide electrostatic or steric stabilization of ZnO nanoparticles and increase their cytotoxic effects. The cytotoxicity and cell death induction were assessed using two human breast adenocarcinoma cell lines (MCF-7 and MDA-MB-468). The MTT results indicated that the toxicity of ZnO nanoparticles was significantly increased upon MTCP conjugation. Annexin/PI and real time RT-PCR results demonstrated that the ZnO-MTCP nanoparticles induced cell death via different non-canonical pathways that are under Ca^{2+} control. Calcium signaling could regulate lysosomal dependent apoptosis and death autophagy, and killing of the two selected types of breast cancer cells.

1. Introduction

Nanotechnology is a new technology and has rapidly improved in recent years. Nanoparticles are used in many industries including engineering, medicine, and cosmetics (Griffitt et al., 2007; Jafarirad et al., 2016). Nanoparticles have larger surface area even though these surfaces may have different physical and chemical properties and named Janus nanoparticles (Han et al., 2016). Two decades of nanotoxicology research has demonstrated that some nanoparticles can be toxic and have lethal effects (Bharali et al., 2009; Salata, 2004). Metal nanoparticles have many application in control of infection (Ashfaq et al., 2016). Metal oxide nanoparticles are the most toxic known nanoparticles, and numerous studies have focused on their toxic effects. Titanium oxide is used as a treatment against cancer cells because these nanoparticles can produce free radicals and induce cell death (Cai

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et al., 1992; Wang et al., 2007). Zinc oxide is also a well-known toxic metal oxide with good potential for cancer therapy (Hu et al., 2009). Zinc oxide nanoparticles are semiconductor nanoparticles with wide band gap (Afzali et al., 2016; Krupa and Vimala, 2016). We hypothesized that stabilization of ZnO nanoparticles will prevent their accumulation and agglomeration, and will increase their cytotoxicity (Alswat et al., 2016).

Two methods exist for preventing unexpected oligomerization of nanoparticles resulting in their stabilization. These include electrostatic and steric stabilization (Tadros et al., 2004). Nanoparticles in their stabilized forms lack the tendency to become agglomerated and exhibit larger surface area leading to the attachment of more killing agents. They can kill any desired cells such as bacteria or cancer cells, target more moieties, lead the nanosystem towards the target cells, and better imaging agent to aid their use in clinical diagnosis (Ahmed et al., 2016; Eastman et al., 2001; Ghaedi et al., 2016; Rath et al., 2016). Cell death induction of different nanoparticles in cancer treatment are studied, and apoptosis is most common (Ahmad et al., 2012; Miura and Shinohara, 2009; Park et al., 2008; Selim and Hendi, 2012; Wang et al., 2014). In our recent work, we showed that conjugation of MTCP to PAMAM and HPMA could change the total positive zeta potential of the nanopolymers proving the electrostatic as well as steric effects of MTCP on the nanopolymers (Mohammadpour et al., 2016). In this current study, we proposed that MTCP conjugation could stabilize ZnO nanoparticles possibly via either electrostatic or steric stabilization resulting in enhanced cytotoxic effects of ZnO in two human breast adenocarcinoma cell lines (MDA-MB-468 and MCF-7) compared with ZnO nanoparticles.

2.0 Materials and Methods

2.1 Materials

The MCF-7 and MDA-MB-468 cell lines were obtained from Iran National Genetic Resources (Tehran, Iran). In order to obtain a better generalized result for clinical use, the selection of these cell lines was performed based on the common classification of breast cancer cells (Badve et al., 2011; Perou et al., 1999; Perou et al., 2000). MCF-7 cells belongs to luminal A group of breast cancer cell lines having low proliferative activity, low degree of malignancy, express estrogen/progestron receptors, and lack Her2 receptor. MDA-MB-468 is categorized in basal or “triple negative” group (ER/PR-negative, HER2 -negative). The RPMI-1640 and DMEM-HAMs F-12 medium (Gibco, USA) were used to culture MCF-7 and MDA-MB-468 cells, respectively. Penicillin-Streptomycin solution, 10% Fetal Bovin Serum (FBS), and Trypsin–EDTA (5X) solution were from Gibco. Dimethylthiazole diphenyltetrazolium bromide (MTT), DMSO, zinc acetate dehydrate, cysteine, EDC, Sulfo-NHS and PI were from Sigma (USA). Annexin-PI kit was purchased from eBiosciences company (USA). Real qPCR kit (Ampliqon Company, Korea) was used for gene expression analysis. Diethylene glycol was from Merck (Germany) and Meso-Tetra (4-Carboxyphenyl) Porphyrin (MTCP) was from Frontier Scientific (USA).

2.2 Methods

2.2.1 Determination of cell viability—MCF-7 (1×10^4) and MDA-MB-468 (7×10^3) cells were seeded in each well of a 96-well plate. MCF-7 cells were cultured in RPMI-1640

medium and MDA-MB-468 cells in DMEM-HAM's F12. After 30–36 hours, when the cells reached 50% confluence they were incubated with freshly prepared medium with different concentrations of ZnO-MTCP (3.7, 5.2, 7.4, 15.8 and 22.2% v/v) for 14 h. This time is essential for the entry of nanoparticles into the cells. Following incubation, the cells were fed with fresh complete medium for another 48 h. To determine cell viability, the medium was replaced and 20 μ l medium containing a filtered solution of MTT (5 mg/ml) was added to each well to allow the conversion of yellow tetrazolium salt into an insoluble purple formazan crystals. Formazan crystals were dissolved in 100 μ l of DMSO, and after 30 min the solution absorbance was obtained by scanning with a Rayto microplate reader (China) at 570 nm. Each experiment was performed at least three times in triplicates.

2.2.2 Synthesis and characterization of the nanoparticles—The synthesis of zinc oxide nanoparticles and its conjugation to MTCP was carried out as previously described with some modifications (Sadjadpour et al., 2016). Briefly, 0.0032 g zinc acetate dihydrate was dissolved in 3 ml of diethylene glycol and stirred under reflux. The temperature was changed from 25°C to 110°C during a 30 min incubation, and then it was raised to 160°C during a 40 min incubation. After cooling, pH of the sample was set to 7.0–7.2 via adding 20 μ l of NaOH solution (5 N). The sample was placed in an ultrasonic bath for 3 min and centrifuged for 15 min at 12,000 rpm. The supernatant containing smaller sized nanoparticles was collected and further characterized.

The nanoparticles were characterized for hydrodynamic radius using dynamic light scattering detector attached to Zetasizer device (Malvern Company, England). The results were plotted as a nanoparticle size distribution curve. FE-SEM was used to verify the size of the nanoparticles in the DLS. The Cold Field Emission Device manufactured by Hitachi, Model: S4160 (Japan) was used and the images were captured at x300,000 magnification.

2.2.3 Synthesis and characterization of Zinc oxide-MTCP conjugates—ZnO nanoparticles have no functional groups for MTCP attachment. Thus, cysteine conjugation was carried out before MTCP conjugation. For cysteine conjugation of ZnO nanoparticles, 15 μ l of cysteine stock solution was added to 1.5 ml of the nanoparticles and sonicated in a ultrasonic bath (Soltec, Italy) for 3 minutes. The solution was then stirred for 2 h at room temperature. Cysteine-conjugated nanoparticles were purified using a 3 kDa Amicon Ultra Centrifugal Filter to remove free non-conjugated cysteines. The pure sample on the filter was then dissolved in 1.5 ml of deionized water. Carboxylic groups of MTCP were activated via a carbodiimide reaction with DCC/NHS. Briefly, MTCP was dissolved in 0.5 ml double distilled water in a final concentration of 0.42 mM and 0.05 mg of Sulfo-NHS, and 0.05 mg of EDC was added to the solution at pH 7–7.4. The solution was stirred for 15 min at room temperature and kept in the dark. Following incubation, 1 ml of the purified cysteine-conjugated nanoparticles was added to the activated MTCP and the sample was stirred for 2 additional hours under high speed. Untreated MTCP was removed using a 3 kDa Amicon Ultra Centrifugal Filter.

Fluorescence spectroscopy was performed using a Cary Eclipse device (Agilnet Company; USA). In order to check the MTCP conjugation, emission spectra of free MTCP and ZnO nanoparticles were recorded before and after cysteine and MTCP conjugation. To carry out

the fluorescence experiments, ZnO-MTCP sample had equivalent concentrations of ZnO and MTCP with the free ZnO and MTCP samples. The difference between the emission spectra of these samples indicated that the cysteine-conjugated nanoparticles were attached to MTCP because the emission peak of ZnO-Cys at 430 nm was absorbed by the conjugated MTCP and its intensity reached zero. However, the emission peak of MTCP appeared at 640 nm indicating a reasonable proximity between MTCP and ZnO-Cys nanoparticles for fluorescence resonance energy transfer (FRET).

2.2.4 Gene expression analysis—RNA extraction was performed using the RNX-plus kit (CinnaGen, Iran) as recommended by the manufacturer. Briefly, cells were detached from the 6-well plates using trypsin-EDTA and were collected after washing with PBS. For each 10^6 cells 200 μ l of the kit reagent was added and after 10 seconds, the samples were vortexed for 10 sec and kept at room temperature for 5 min. Following incubation, 40 μ l of chloroform was added, mixed for 15 seconds, put on ice for 5 min, and centrifuged at 12,000 rpm for 15 min at 4°C. Upper phase was separated slowly and the same volume of cold isopropanol was added to precipitate RNA after 15 min of incubation on ice. Precipitated RNA was first isolated by centrifugation at 4°C under 12,000 rpm for 15 min, and then washed with 200 μ l of cold 75% ethanol, and precipitated again by centrifugation (10 min at 4°C under 7,500 rpm). Finally, the washed RNA pellet was dissolved in 20 μ l of DEPC water and used for further analysis. The quantity and quality of the RNA were determined using a Nanodrop (Thermo Scientific 2000c, USA)

Synthesis of cDNA was carried out using a cDNA synthesis kit (Ariyatous, Iran) using 1 μ g of total RNA and random hexamer as recommended by the supplier. The following program: 10 min at 25°C, 60 min at 50°C, and 10 min at 70°C, was used. The cDNA samples were then stored at -20°C for later use. Real time qPCR SYBER Green kit (Ampliqon, Korea) was used for gene expression studies. The PCR reaction was performed in the final volume of 10 μ l consisting of 5 μ l of the main solution, 1 μ l of cDNA, 1 μ l of forward and reverse primers, and 3 μ l of DEPC water. The temperature program used to perform PCR was as follows: initial denaturation at 95°C for 15 min, 40 amplification cycles containing 15 sec at 95°C, 30 sec at 60°C and 1 min at 72°C. The PCR was carried out using an ABI device (Step one). In each PCR, a sample containing forward and reverse primers with no cDNA was also used to ensure proper calculations.

2.2.5 Cell cycle analysis—For cell cycle distribution studies, cells were cultured in 6-well plates and allowed to reach 50% confluence. Cells were then incubated with concentrations of nanoparticles that caused 40% and 60% cellular death (LC₆₀ and LC₄₀, respectively), 9% and 13% (v/v) ZnO-MTCP for MCF-7 and 4% and 6% (v/v) for MDA-MB-468 cells, for 14 h. Following incubation, cells were refed with fresh medium and incubated for an additional 48 h. Cells were then trypsinized and collected by centrifugation (5 min at 3,500 rpm), washed with PBS, and the resulting cell pellet was collected for staining. In order to stain, dye solution consisting of propidium iodide (5 μ g/ml) and RNase (10 μ g/ml) (Ariyatous, Iran) were prepared in PBS. RNase was used removed false-positive results, which is derived from propidium iodide binding to RNA. The dye solution (500 μ l) was added to the cell pellets containing approximately 1–1.5 million cells and kept in the

dark for 45 min at room temperature. The stained cells were then analyzed in a flow cytometer (BD FACSCallibur, USA).

2.2.6 Cell death studies—In order to determine the percentage of apoptosis and necrosis, cells were stained with AnnexinV/PI (eBioscience). Cells were cultured in a 6-well plate and allowed to reach 50% confluence before incubation with different concentrations of nanoparticles to induce 40% (LC₄₀) and 60% (LC₆₀) cell death. After 14 h, cells were fed with fresh medium and incubated for an additional 48 h. Cells were then harvested and washed. Cell pellets with approximately 1–1.5 million cells were resuspended in 200 µl of binding buffer provided by the kit. The obtained cell suspension was divided to two parts and transferred to two separate vials. Annexin-FITC dye (5µl) was added to one of the vials and after the incubation time, each vial was analyzed using a flow cytometer. PI dye (5 µl) was added to the first and second vials and they were analyzed by a flow cytometer. The data analysis was performed using FLOWJo software.

3.0 Results

3.1 Size of ZnO-MTCP nanoparticles

The synthesis of zinc oxide nanoparticles and their conjugation with MTCP was performed based on our previous work with some modifications (Sadjadpour et al., 2016). The size of nanoparticles was studied by DLS. These results indicated that in solution state the particle size ranged between 10 to 25 nm. This was further confirmed by FE-SEM analysis indicating the existence of a homogeneous spherical nanoparticle with average size of 20 to 30 nm (Figs. 1 and 2).

3.2 Study of ZnO conjugation to cysteine and MTCP using fluorescence spectroscopy

Conjugation of cysteine to ZnO nanoparticles was verified from the decreasing intensity of emission at 430 nm (Fig. 3). Conjugation of cysteine to ZnO nanoparticles caused no significant structural changes in nanoparticles since the absorption spectrum of ZnO-Cys showed minimal differences with the absorption spectrum of ZnO nanoparticles (Figure 3). Binding of MTCP to cysteine-conjugated MTCP can be documented by vanishing of the emission ZnO peak around 400 nm and rising of the two emission peaks around 640 nm, which relates to MTCP (Fig. 3). These results confirmed the successful conjugation between MTCP and ZnO nanoparticles resulting in adequate proximity between these two moieties for FRET (Fig. 3). At the time of FRET, the photons emitted by the nanoparticles at 430 nm can be absorbed by the conjugated MTCP, and instead MTCP-emission photons are emitted at 640 nm.

3-3 Cytotoxicity of ZnO-MTCP nanoparticles

The cytotoxicity of ZnO-MTCP nanoparticles was assessed using the MTT assay. The results were obtained using different concentrations of ZnO-MTCP (3.7, 5.2, 7.4, 15.8 and 22.2% v/v). These were prepared by adding 5, 7, 10, 20 and 30 µl of ZnO-MTCP to the medium in the final volume of 130 µl (Fig. 4). Viability study of MCF-7 cells incubated with ZnO-MTCP indicated a LC₅₀ of around 15% (v/v) concentration. Similar lethality rate was

obtained for MDA-MB-468 cells at approximate ZnO-MTCP concentration of 7.5% (v/v) indicating a more sensitive state for these cells compared with MCF-7 cells (Fig. 4).

To ensure that the cytotoxic effects of ZnO-MTCP are related to the stabilization role of MTCP exerted on ZnO nanoparticles, the viability of MCF-7 and MDA-MB-468 were determined in the presence of equivalent amounts of ZnO, MTCP and ZnO-MTCP (Figs. 5 and 6). We observed that conjugation of MTCP to ZnO nanoparticles augmented the cytotoxic potency of ZnO-MTCP nanoparticles compared with free ZnO or MTCP alone.

In order to study the cellular effects of ZnO-MTCP on breast cancer cells, two effective concentrations resulting in 40% and 60% cell death (i.e. LC₄₀ and LC₆₀) were selected. These concentrations were utilized for gene expression analysis, cell cycle investigations, and flow cytometric determination of the types of cell death. The 40% and 60% induction of cell death was obtained at 9% and 13% (v/v) ZnO-MTCP concentration for MCF-7 cells, and 4% and 6% (v/v) ZnO-MTCP concentration for MDA-MB-468 cells.

3.4 Cell cycle analysis

In order to investigate the cell cycle changes, simultaneous treatment of propidium iodide and RNase A was used. Figs. 7 and 8 represent the results of cell cycle studies in MCF-7 and MDA-MB-468 cells at LC₄₀ and LC₆₀ concentration of ZnO-MTCP. The cell cycle arrest in MCF-7 cells was observed at the S phase, and its intensity was increased at the higher concentration. The cell cycle arrest in MDA-MB-468 cells was observed mainly in S phase for LC₄₀ and in S and G2 phases for LC₆₀ concentrations.

3.5 Studies of cell death using flow cytometry

In analysis of flow cytometry data, the determination of cell population distribution was performed using two-dimensional plot of FSC against SSC (not shown). The areas of the four Q₁-Q₄ regions were determined based on FSC/SSC plots of the control groups using FlowJo software. The divided Q₁₋₄ areas showed the percentage of necrotic cells, old apoptotic cells, young apoptotic cells, and normal cells, respectively (Figs. 9 and 10).

Both MCF-7 and MDA-MB-468 cells were severely affected by apoptotic death in the presence of ZnO-MTCP nanoparticles (Figs. 9 and 10). Induced cell death in MCF-7 cells was mainly late apoptosis (cell distribution is mainly in region of Q₂), whereas in MDA-MB-468 cells the induced cell death was early apoptosis showing cell distribution mainly in the Q₃ region.

3.6 Real time RT-PCR analysis

To determine the mechanism of apoptosis induction in these cells, real time RT-PCR was performed for some key genes with effective roles in apoptosis, autophagy and cell survival pathways. In apoptosis pathway, Ct values of *bax*, *bcl2*, *p53*, *aif* and *casp3* genes was compared with the Ct value of *gapdh* as the reference gene. This was also performed for cell survival (*akt1*, *mTor*, *pten*) and autophagy genes (*beclin1*, *lc3*, *atg5*, *dram*). The results of gene expression studies for selected genes in MCF-7 and MDA-MB-468 cells are shown in Figs. 11 and 12.

4.0 Discussion

In recent decades ZnO nanoparticles, because of their various functions, are widely used in biosensors, cosmetics, food supplements, and drug designs. However, ZnO nanoparticles are considered as one of the most toxic nanoparticles (Huang et al., 2010; Lanone and Boczkowski, 2006; Rasmussen et al., 2010; Sharma et al., 2009). These nanoparticles have different toxic effects on cells that can be mainly attributed to their oxidizing effects and the resulted damage driving cell death (Deng et al., 2009; Zhao et al., 2009). The most important damage of metal oxide nanoparticles is oxidative stress, and has been investigated in both in vitro and in vivo conditions (Guo et al., 2013a; Guo et al., 2013b; Sharma et al., 2012; Shukla et al., 2011). This oxidative stress is caused by a misbalance between ROS production and contrasting effects of antioxidative compounds (Limón-Pacheco and Gonsebatt, 2009). ROS can damage cellular proteins leading to stress signaling and cell death (Azad et al., 2009; Brigelius-Flohé, 2009; Wang et al., 2014; Wu, 2006).

ZnO nanoparticles were synthesized here using Cheng et al. sol-gel method with some modifications (Cheng et al., 2006). Characterization of nanoparticles was ascertained using FE-SEM and DLS, and demonstrated that the size of the nanoparticles was 30 ± 3 nm and their morphology was spherical (Figs. 1 and 2). After MTCP conjugation, attachment of MTCP to ZnO nanoparticles was substantiated based on the presence of an emission peak around 640 nm in ZnO-MTCP spectrum (Fig. 3). The lack of this peak in ZnO fluorescence spectrum, and its increase in the spectrum of ZnO-MTCP nanoparticles depicted the occurrence of FRET between the ZnO and its bound MTCP. Moreover, waning of the absorbance peak of ZnO around 370 nm in ZnO-MTCP spectrum also occurred in parallel with the rising of the emission peak around 640 nm. These observations supported the existence of an effective FRET between MTCP and ZnO nanoparticles (Fig. 3).

To investigate the cytotoxicity of ZnO-MTCP nanoparticles, the MTT colorimetric assay was carried out (Fig. 4). Comparison of the lethality of free ZnO and ZnO-MTCP nanoparticles demonstrated that MTCP conjugation resulted in more cytotoxicity. This is possibly achieved by electrostatic or steric stabilization of the nanoparticles (Figs. 5 and 6). Stabilization of ZnO nanoparticles is performed in possible applications of ZnO nanoparticles as wound dressing materials (Chaturvedi et al., 2016). The cell cycle analysis of MCF-7 cells incubated with ZnO-MTCP demonstrated an increase in the percentage of cells in S phase compared with controls (Fig. 7). The ZnO nanoparticles could arrest the cell cycle in S and G2/M phases in RGC-5 cells (Guo et al., 2013b). Here cellular behavior against ZnO nanoparticles indicated the presence of a dose dependent criterion, such that at higher concentration the percentage of the cells in S phase was increased (Fig. 7). It is reported that cell cycle arrest at the DNA synthesis step (S phase) can occur as a result of DNA damage (Ye et al., 2003). Thus, ZnO-MTCP nanoparticles could also affect DNA health leading to impaired DNA synthesis and cause cell cycle arrest in the S phase. In MDA-MB-468 cells, cell cycle arrest occurred in S phase with the two concentrations of ZnO-MTCP nanoparticles used here (Fig. 8). We proved here that the possible mechanism for induced death in cells incubated with ZnO-MTCP may originate from the increasing of calcium ion concentration in the cytosol (see below). It is reported that during Ca^{2+} elevation in the cytosol, nuclear lamina degradation (Oberhammer et al., 1994; Rao, 1996; Rao et al.,

1996), DNA fragmentation (Pandey et al., 1994; Walker et al., 1994) and activation of resident caspases in ER (Nakagawa and Yuan, 2000) may occur. Thus, the given DNA fragmentation in the presence of increased cytoplasmic Ca^{2+} is a good indication of the observed S phase arrest (Figs. 7 and 8).

To study the percentage of necrosis and apoptosis cell death, flow cytometry was carried out using Annexin-FITC and PI staining (Figs. 9 and 10). The results in MCF-7 and MDA-MB-468 cells showed that apoptosis induction occurred at about 80% at both concentrations of ZnO-MTCP evaluated. However, based on the MTT results in the two concentrations of ZnO-MTCP only 40% and 60% of cell death was registered (compare Fig. 4 with Figs. 9 and 10). Since in some of the early apoptotic cells the mitochondrial activity of succinate dehydrogenase, which is responsible for the colorogenic reaction in MTT assay, is not fully lost. Thus, this category of the dying cells are registered as live cells in MTT assay. The incidence of 80% of apoptotic death in both concentrations of ZnO-MTCP in MCF-7 and MDA-MB-468 cells can be firstly caused by DNA damage leading to the cell cycle arrest in S phase followed by induction of cell death through apoptosis (Figs. 7–10). Induction of cell death after S phase arrest is also reported by Iguchi and Ostroff (Iguchi et al., 2007).

The expression level of some apoptotic genes was determined using real time RT-PCR. These results indicated that the incubation of MCF-7 cells with nanoparticles resulted in a significant increase in the expression of *bcl2* that paralleled with a decrease in *bax*, *p53* and *aif* proapoptotic genes (Fig. 11). The observed reduction in *p53* expression is one of the factors responsible for a dramatic increase in *bcl2* expression, as much as 12-fold, because *bcl2* gene is normally under a negative control by p53 (Findley et al., 1997; Hemann and Lowe, 2006). With respect to the controlling role of p53 on *bax* gene (Yang et al., 2004), the subsequent declining of *bax* after p53 is also expected (Fig. 11). Decreased expression of *bax* and *aif* and the increased expression of *bcl2* support the lack of canonical apoptosis and presumably triggering of non-canonical apoptosis and cell death pathways.

Incidence of cell death under decreasing of *bax* expression is reported to support the more sensitized form of apoptosis in *bax* deficient cells (Findley et al., 1997; Pecorino, 2012; Shimizu et al., 2004). Bcl2 is an anti-apoptotic protein that normally supports cell survival when its cellular concentration is normal. However, there are some reports indicating that cells with overexpressed state of *bcl2* are more sensitive to apoptosis, like death autophagy and it can be induced by increased cytosolic Ca^{2+} (Anderson et al., 2002; Shimizu et al., 2004). Various anticancer drugs can activate autophagy cell death in breast cancer cells including sulfadiazine (Mohammadpour et al., 2012). Bcl2 in an unstressed condition has its normal cytosolic concentration for decreasing IP3-mediated Ca^{2+} release to result in calcium accumulation, preferably, in the ER (Lam et al., 1994). Under this unstressed condition, only some part of the cytosolic calcium could enter into the mitochondria to maintain the shape and structure of the organelle leading to prevention of pro-apoptotic agents leakage and resist cell death (Marin et al., 1996; Pinton et al., 2001). This is the reason for anti-apoptotic effects of Bcl2 under normal expression to prevent cell death induction, as occurs in ceramide induced apoptosis. This type of apoptosis occurs via increasing of the cytosolic calcium and prolonged existence of Ca^{2+} in the mitochondria results in organelle deformation and releasing of pro-apoptotic agents (Pinton et al., 2001). Thus, antagonistic

effect of Bcl2 against ceramide is evoked by its decreasing effects on IP3 receptors on the ER membrane to adjust the critical concentration of calcium ions in the cytosol.

In contrast to the anti-apoptotic effects of Bcl2, there are several reports indicting apoptotic potential of Bcl2 (Lithgow et al., 1994; Minn et al., 1997; Schendel et al., 1997). Apoptotic effects of Bcl2 are evoked under cellular stress when it is overexpressed. The Bcl2-membrane oligomerization in the ER results in increased calcium release in the cytosol and mitochondria (Minn et al., 1997; Schendel et al., 1997). Ca^{2+} overload is a potent stimulus for opening of permeability transition pores (PTP) in the mitochondria (Bernardi et al., 1998; Csordás et al., 1999; Rizzuto et al., 1998) or increasing the lysosome membrane permeability (LMP) resulting in efflux of the lysosomal enzymes such as calpains or the phosphatase calcineurin (Boya and Kroemer, 2008; Clarke et al., 2012; Squier et al., 1999). Lysosomal leakage also occurs for some endonucleases to cause DNA damage, and for some proteases like cathepsins to promote apoptosis (Zhu and Loh, 1995).

In treated MCF-7 cells following overexpression of *bcl2* and the declined expression level of *atg5* and *beclin1*, the two important agents of canonical autophagy, was observed in parallel with the increase in *lc3* expression (Fig. 11). Bcl2 can block the positive effects of Beclin1 for induction of canonical autophagy (Ciechomska et al., 2009; Erlich et al., 2007), whereas under its overexpression autophagy can be triggered via different non-canonical pathways. The stress-induced death autophagy is independent of apoptosis (Xiong et al., 2015), while they are under Ca^{2+} control and the Ca^{2+} channel, IP_3R , plays a crucial role in calcium-ERK-mediated autophagy and calcium mitochondria-caspase-induced apoptosis (Berridge et al., 2000; Wang, 2008). There are also some reports that show most of the compounds applied for Ca^{2+} -mediated autophagy also promote apoptosis (Gastaldello et al., 2010; Høyer-Hansen et al., 2007; Law et al., 2010). Therefore, it appears that overexpression of *bcl2* in our treated MCF-7 cells could concurrently induce apoptosis and autophagy via Ca^{2+} signaling pathways.

Alternatively, increased *bcl2* expression occurred along with a reduction in *akt* expression in the lower concentration of ZnO-MTCP (Fig. 11). Akt is a kinase which could recruit PI3-kinase toward the plasma membrane to phosphorylate a series of downstream proteins such as FOXO, Bad and Caspase-9 to prevent their activity and inhibit cell death (Cardone et al., 1998; Datta et al., 1997). In addition, Akt causes deactivation of NF- κ B and activation of cFLIP, and thereby inhibits apoptosis (Ozes et al., 1999). In our studies, we suggest that the reduction of *akt* expression is pursued by declining of its inhibitory effect on apoptosis to initiate cell death in the form of the above mentioned lysosome-dependent pathway. It is also reported that removing of Akt inhibitory effects on caspase-9 is able to induce cell death in the lysosome dependent manner (Gyrd-Hansen et al., 2006). This reported mechanism for Akt is in accordance with the Bcl2 induced mechanism of cell death in relation to increased cytosolic calcium and induction of lysosome-dependent cell death.

In MDA-MB-468 treated cells, we observed that the expression of *p53* and *aif* genes were increased (Fig. 12). In addition, we know that in MDA-MB-468 cells all allelic forms of p53 are R273H mutated, but they still are active to induce transcription of some specific genes (Chen et al., 1993; Nigro et al., 1989). The mutant form of p53 in this cell line has the ability

to bind to the promoter of those genes which are not under the control of native p53, for example, *map2k3* (Gurtner et al., 2010). Increased expression of *map2k3* causes cell senescence leading to the cell cycle arrest (Gurtner et al., 2010; Jia et al., 2010; van Doorn and Woltering, 2004). Thus, induction of cell senescence is a good candidate to explain the induced cell cycle arrest in MDA-MB-468 when they were incubated with ZnO-MTCP nanoparticles (Fig. 8). Our findings also indicated that Bcl2-associated cell death is the main mechanism for cell death induction in treated MDA-MB-468 cells. We observe that *bcl2* gene expression was dramatically increased (Fig. 12), which may occur because of the observed increase in the expression of mutated *p53* in MDA-MB-468 cells. As mentioned before, the increased Bcl2 expression leads to increased cytosolic calcium ions and release of lysosomal enzymes inducing the lysosome-dependent cell death (Gyrd-Hansen et al., 2006).

In MDA-MB-468 treated cells, *aif* gene expression, similar to Bcl-2, was increased (Fig. 12). AIF is a protein located between the two mitochondrial membranes (Susin et al., 1999). When AIF is released into the cytosol, it triggers caspase-independent apoptotic death via DNA fragmentation (Candé et al., 2002). In fact, in MDA-MB-468 treated cells, in addition to the increased expression level of *bcl2* and its effects on the calcium-dependent lysosomal death, the caspase-independent apoptotic death, induced by AIF, (AIF dependent apoptosis) was also another auxiliary mechanism that helps calcium ions to induce cell death (Mohammadpour et al., 2014).

Our gene expression studies indicated that the autophagic genes such as *atg5*, *beclin1* and *lc3* were increased in MDA-MB-468 treated cells (Fig. 12). This increasing pattern of expression was not observed in MCF-7 treated cells (Fig. 11). The three cited genes are the most important genes in classical autophagy pathway (He and Levine, 2010; Marquez and Xu, 2012; Oberstein et al., 2007). Autophagy is a 2-phase process that exerts both tumor suppressive and prosurvival effects. Tumor suppressive effect of autophagy where Akt is inhibited is called type II programmed cell death (Mohammadpour et al., 2013). There are some reports that indicate severe relationship between cytosolic calcium ion concentration and death autophagy which mainly is triggered due to the activation of CAMKK α kinase (Høyer-Hansen et al., 2007; Høyer-Hansen and Jäätelä, 2007). Thus, our quantitative gene expressions studies indicate that the observed apoptosis in MDA-MB-468 cells depends on calcium ion signaling and lysosomal-dependent autophagy cell death.

5.0 Conclusions

MTCP conjugation of ZnO nanoparticles may cause their electrostatic or steric stabilization and inhibition of agglomeration. This resulted in increased surface area causing greater cytotoxicity, and has an excellent potential for clinical applications in cancer treatment. The cancer cells originate from normal cells, and there are many similarities between a cancer cell and a normal cell. With respect to this notion, treatment of cancer cells is very difficult since cytotoxic effects of the drug will also occur in the normal cells. The binding or trapping of a drug in a carrier such as nanoparticles and use of monoclonal antibodies, aptamers or folic acid for targeted therapy will overcome this limitation. This will allow delivery of the drug individually toward the cancer cells, and it is our main future objective.

We saw here that cytotoxic ability of ZnO-MTCP nanoparticles in MCF-7 and MDA-MB-468 cells was mediated through the non-canonical apoptotic cell death. Apoptotic death was presumably induced by increasing of the cytosolic calcium resulting in lysosomal and autophagy dependent cell death

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Fig. 1. Determination of the nanoparticle size by DLS

The DLS results indicated that the main population of synthesized zinc oxide nanoparticles have diameters in the range of 10 to 25 nm.

Fig. 2. Determination of the size and shape of the nanoparticles using Field Emission Scanning Electron Microscopy

This picture shows the existence of zinc oxide particles with an approximate diameter of 20 to 30 nm. The scale bar is placed on the left-bottom side of image. The applied voltage was 1500 kV.

Fig. 3. The emission spectrum of the free, cysteine-, and cysteine and MTCP-conjugated nanoparticles

In this figure, the solid line, dashed line, dotted line and solid-dotted line represent the emission spectra of ZnO, ZnO-Cys, diluted solution of ZnO-Cys and ZnO-Cys-MTCP, respectively. Excitation wavelength for recording emission spectra was 320 nm. Elimination of the maximum emission of ZnO-Cys at 430 nm and the appearance of maximum emission around 640 nm demonstrate the successful conjugation of MTCP to nanoparticles and the occurrence of FRET between MTCP and ZnO nanoparticles.

Fig. 4. The viability of MDA-MB-468 and MCF-7 cells incubated with various concentrations of ZnO-MTCP

In this histogram, * and ** symbols represent statistical significant difference between the samples incubated with ZnO-MTCP and untreated controls in statistical levels of $p < 0.05$ and $p < 0.01$, respectively.

Fig. 5. The viability of MCF-7 cells in the presence of equal amounts of ZnO, MTCP and ZnO-MTCP

In this diagram, the abbreviations of ND and NP are used for Nanodevice (ZnO-MTCP) and Nanoparticle (ZnO). Comparing the height of the black columns with the gray ones, especially at the higher concentrations, shows a strong promotion of cytotoxic effects of ZnO when it is conjugated with MTCP. * and ** symbols represent the statistical significant difference between the treated samples compared with the untreated controls in the statistical levels of $p < 0.05$ and $p < 0.01$, respectively. Statistical difference between ND and NP or between ND and free MTCP is also shown using the “L” shape symbol.

Fig. 6. The viability of MDA-MB-468 cells in the presence of equal amounts of ZnO, MTCP and ZnO-MTCP

In this diagram, the abbreviations of ND and NP are used for Nanodevice (ZnO-MTCP) and Nanoparticle (ZnO). Comparing the height of the black columns with the gray ones, especially at the higher concentrations, shows a strong promotion of cytotoxic effects of ZnO when it is conjugated with MTCP. * and ** symbols represent the statistical significant difference between the treated samples compared with the untreated controls in the statistical levels of $p < 0.05$ and $p < 0.01$, respectively. Statistical difference between ND and NP or between ND and free MTCP is also shown using the “L” shape symbol.

Fig. 7. The cell cycle analysis of MCF-7 cells incubated with two different concentrations of ZnO-MTCP (LC₄₀ and LC₆₀)

In the right histogram, * and ** symbols represent the significant difference between the treated samples and untreated controls in statistical levels of $p < 0.05$ and $p < 0.01$, respectively. Statistical difference between the results of LC₄₀ and LC₆₀ is also shown in the histogram using the “L” shape symbol.

Fig. 8. The cell cycle analysis of MDA-MB-468 cells incubated with two different concentrations of ZnO-MTCP (LC₄₀ and LC₆₀)

In the right histogram, * and ** symbols represent the significant difference between the treated samples and untreated controls in statistical levels of $p < 0.05$ and $p < 0.01$, respectively. Statistical difference between the results of LC₄₀ and LC₆₀ is also shown in the histogram using the “L” shape symbol.

Figure 9. The study of cell death in MCF-7 cells incubated with two different concentrations of ZnO-MTCP (LC₄₀ and LC₆₀)

** symbol represents significant difference between the treated samples and the untreated controls in statistical level of $p < 0.01$.

Figure 10. The results of cell death studies in MDA-MB-468 cells incubated with two different concentrations of ZnO-MTCP (LC₄₀ and LC₆₀)

** symbol represents significance difference between the treated samples and untreated controls in statistical level of $p < 0.01$. Statistical differences between the results of LC₄₀ and LC₆₀ is also shown in the histogram using the “L” shape symbol.

Fig. 11. Real time RT-PCR determination of gene expression in MCF-7 cells incubated with two different concentrations of ZnO-MTCP (LC₄₀ and LC₆₀)

* and ** symbols represent the significance difference between the treated samples and the untreated controls in statistical levels of $p < 0.05$ and $p < 0.01$, respectively. Statistical differences between the results of LC₄₀ and LC₆₀ is also shown in the histogram using the “L” shape symbol.

Fig. 12. Real time RT-PCR determination of gene expression in MDA-MB-468 cells incubated with two different concentrations of ZnO-MTCP (LC₄₀ and LC₆₀)

* and ** symbols represent the significance difference between the treated samples and the untreated controls in statistical levels of $p < 0.05$ and $p < 0.01$, respectively. Statistical differences between the results of LC₄₀ and LC₆₀ is also shown in the histogram using the “L” shape symbol.