Deregulation of *BCL2* family genes in glioblastoma cells consequent to poly(butyl cyanoacrylate) nanoparticles treatment

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Brain tumours develop resistance to chemotherapy mainly due to the presence of the blood brain barrier which partially blocks the penetration of drugs into the brain. Poly(butyl cyanoacrylate) nanoparticles have been extensively proposed for delivering drugs into the brain and have shown their ability to cross the blood brain barrier in vitro and in vivo. However, the application of nanoparticles could be limited by their potential side effects. In this study, two glioblastoma cell lines with the poly(butyl cyanoacrylate) nanoparticles were treated at different concentrations. Through MTT assay, the viability of treated cells was quantified. Induction of apoptosis was analysed by flowcytometry as well as quantitative PCR assay for the expression of *BCL2* family genes. The results of this study showed that the treatment with poly (butyl cyanoacrylate) nanoparticles induced neither apoptosis nor necrosis in these cells. The quantitative real-time PCR results showed that poly(butyl cyanoacrylate) nanoparticles treatment caused significant changes in the expression of some *BCL2* family members. In conclusion, it seems that poly(butyl cyanoacrylate) nanoparticles treatment at non-toxic dose can change the expression of some apoptosis-related genes in glioblastoma cells while has no significant effect on their viability.

1. Introduction: Glioblastoma multiform is the most frequent malignant brain tumour leading to death generally within 12–24 months post-diagnosis [1]. However, glioblastoma is commonly resistant to systemic chemotherapy so that only modest therapeutic success has been achieved so far [2]. Brain tumours develop resistance to chemotherapy mainly due to presence of the blood brain barrier (BBB) which blocks penetration of drugs into the brain [3–5]. Thus, for an effective treatment, many drugs need to be administered in high doses which may cause severe side effects in other organs [6, 7].

Several approaches have been developed to overcome limited access of drugs to the brain [8–11]. One of the most attractive methods is the use of biodegradable polymeric nanoparticles (NPs) as drug carriers [7, 12]. Poly(butyl cyanoacrylate) (PBCA), a biodegradable, biocompatible and minimally toxic polymer, has been proved to be promising candidate for CNS drug delivery [11, 13–16]. It has been reported that, PBCA NPs induced a reversible opening of the BBB. Due to this effect, PBCA NPs might not only be employed as drug carriers but also as specific openers of the BBB. In fact, instead of incorporating drugs into the NPs, drugs may cross the BBB after being simultaneously applied with PBCA NPs [6, 17, 18].

However, advances in utilisation of NPs in medicine can be limited by potential side effects of nanomaterials [19–21]. Most studies that investigated the toxicity of NPs in vitro, have examined cell viability (e.g. by MTT assay) to evaluate the safety of NPs for clinical applications [22, 23]. Though, to further examine the toxicity of nanomaterials, their different aspects of cellular and molecular effects must be investigated. Particularly, apoptosis induction, which is critically important in tumour pharmacotherapy, should be evaluated [20, 24, 25].

The objective of present study was to test the toxicity of PBCA NPs on two glioblastoma cell lines through cell viability assay and gene expression analysis. It is an important step to find out the effects of PBCA NPs (as important carriers for CNS drug delivery) on glioblastoma cell lines from cellular and molecular aspects. This can pave the way for further studies, which focus on comparison of the efficiency of free and NP-loaded anticancer drugs in treatment of glioblastoma as the most frequent brain tumour.

2. Methods: Using emulsion polymerisation, the PBCA nanospheres were synthesised. Briefly, 1%(v/v) of butylcyanoacrylate was added dropwise to 1% (w/v) Dextran 70 solution (in 0.001 N HCl). The polymerisation was performed for 4 h under constant stirring at 400 rpm and the mixture was neutralised and freeze-dried to obtain powder. Zeta potential and particle size of NPs were measured by dynamic light scattering (HSA3000, Malvern, UK) and the morphological examination of PBCA nanospheres was performed using scanning electron microscope (SEM) (XL30, Philips, Netherlands).

The human glioblastoma cell lines (U87MG and A172) used in the current study were purchased from National Cell Bank of Iran. Cell lines were maintained in growth medium (RPMI-1640) supplemented with antibiotics (penicillin 100 U/ml, streptomycin 100 µg/ml) and foetal bovine serum (10%) under standard culture conditions. Cells in the exponential phase of growth were used. The cytotoxicity of PBCA was tested with MTT assay as demonstrated with detail previously [26]. Cell growth inhibition assay performed with different concentrations of PBCA NPs and for different period of time. The effects of NPs were compared to control cells receiving culture medium without PBCA NPs.

Based on these preliminary experiments, the non-toxic concentration of PBCA NPs was determined as 100 µg/ml for different times (24, 48, 60 and 72 h). Thus, the cell lines were treated with 100 µg/ml of PBCA NPs for 48 h to perform apoptosis analysis and gene expression study. Untreated control cells were also analysed in parallel to the test experiments.

Apoptosis was determined by flowcytometry after both adherent and floating cell nuclei were coloured with propidium iodide (PI) and annexin V-FITC and their signals were detected at 630 and 525 nm, respectively. The data was analysed with FloMax software.

Total RNA was extracted using RNX kit (CinnaGen). Extracted RNA samples with spectrophotometrically high quality (A260/280≥1.8) were selected for cDNA synthesis. RNA was reverse-transcribed with RevertAidTM First-Strand cDNA

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Table 1 Primer sequences used for the amplification of *TBP*, *BCL2*, *BCLXL*, *BCL2L12*, *BAX*, *BAD* and *BAK* genes

Gene	Prime	
	Forward primer	Reverse primer
TBP	AATCATGAGGATAAGAGAGCCACG	AGTCTGGACTGTTCTTCACTCTTGG
BCL2	TGTGGATGACTGAGTACCTGAACC	CAGCCAGGAGAAATCAAACAGAG
BCLXL	GCCACTTACCTGAATGACCACC	GCATTGTTCCCATAGAGTTCCAC
BCL2L12	CAGCTACTCCAGACTTCTATGCTTTG	CAGTATGGCTTCCTTCTCTGTCG
BAX	TTGCTTCAGGGTTTCATCCAG	AGCTTCTTGGTGGACGCATC
BAD	GCAAGCATCATCGCCAGG	CAGCGCCTCCATGATGG
BAK	TCACCTTACCTCTGCAACCTAGC	TGGTCTGGAACTCTGAGTCATAGC

Synthesis kit (Fermentas) according to kit protocol as mentioned elsewhere [26].

Primers for the real-time quantitative polymerase chain reaction (PCR) assay are indicated in Table 1. Gene sequences were downloaded from GenBank website (http://www.ncbi.nlm.nih.gov/Genbank) and primer sequences were designed and checked using Primer Express v.3.0 and Gene Runner software, respectively, and also attested with BLAST website (http://www.ncbi.nlm.nih.gov/tools/primerblast/) to check any unwanted PCR product amplification. Primers for each gene were located on different exons or exon-exon boundaries to avoid amplification from any possible contaminating genomic DNA. The melt curve analysis was performed to assure that only a single PCR product was amplified. TATA-box binding protein (*TBP*) gene was used as reference gene for the real-time quantitative PCR assay. Among seven housekeeping genes which are frequently used as reference genes, *TBP* has been validated for expression studies in glioblastoma multiform tissue [27]. The real-time quantitative PCR experiments were carried out on Rotor-Gene 6000 (Corbett Research) with SYBR Premix Ex. Each assay experiment was performed at least in triplicate and the average C_T values were analysed. Non-template controls (NTCs) were included in each assay. Relative quantity of target genes normalised to *TBP* gene (the reference gene) was determined using the comparative ΔC_T method using the equation: relative gene dosage ratio $= 2_T^{-\Delta\Delta C}$, where $\Delta\Delta C_T = [mC_T \text{ target gene} - mC_T \ TBP]_{\text{test sample}} - [mC_T \text{ target gene} - mC_T \ TBP]_{\text{control sample}}$ [28].

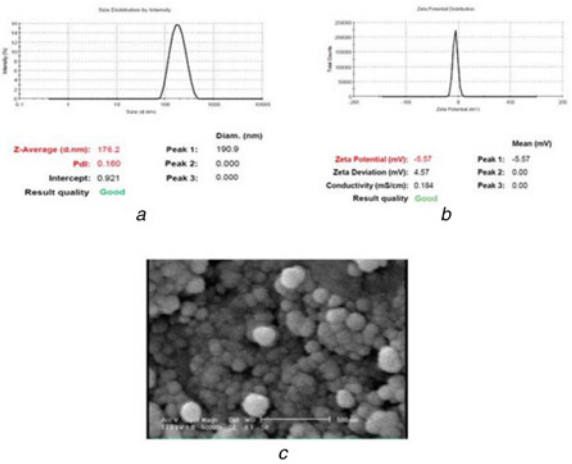

Fig. 1 *PBCA NPs analysed by dynamic light scattering and electron microscope scanning*
a Average size distribution of PBCA NPs detected with dynamic light scattering is about 176.2 nm
b Average surface charge of PBCA NPs detected with dynamic light scattering is about −5.47
c Electron microscope scanning showed that PBCA NPs were completely spherical

For each gene, standard curve was obtained, using diagrammed C_T values against log of five serial two-fold dilutions (75–1200 ng/µl) of control cDNA. The slants of these diagrams were calculated and the PCR efficiency were analysed with the formula: $E = [10^{(-1/\text{slop})} - 1]$ [29]. Agarose gel electrophoresis method was performed and coloration with ethidium bromide was done to inspect the primers' preciseness and producing correct PCR products. The statistical analyses were done with Excel software (Office 2010).

3. Results: PBCA NPs were synthesised by emulsion polymerisation. Dynamic light scattering determined the average size for PBCA NPs about 176.2 nm (Fig. 1*a*). Using zeta potential analysis, pure PBCA nanospheres showed surface negative charge of − 5.57 mV(Fig. 1*b*). Also, SEM study showed that the NPs consisted of distinct spherical particles (Fig. 1*c*).

As MTT assay results determined, relative to the control, PBCA NPs treatment within 100 µg/ml concentration (3.125, 6.25, 12.5, 25, 50 and 100 µg/ml) for 48 h did not decrease the viability of glioblastoma cell lines (Fig. 2*a*). Results also showed, relative to the control, PBCA NPs treatment within 100 µg/ml concentration (50 and 100 µg/ml) for 60 and 72 h did not decrease the viability of glioblastoma cell lines (Fig. 2*b*).

Flowcytometry was used to analyse apoptosis, after treating the cells with 100 µg/ml of PBCA NPs for 48 h. The results were compared with untreated control cells. Signal from FITC/annexin V was measured at 525 nm (FL1 channel) and red signal from PI was measured at 630 nm (FL3 channel). One of the earlier events of apoptosis is translocation of membrane phosphatidylserine (PS) from inner to the outer leaflet of the plasma membrane. Annexin V has high affinity for PS. Viable cells with intact membranes exclude PI, whereas the membranes of dead and damaged cells are permeable to PI. Therefore, $PI^{(-)}$ annexin $V^{(+)}$ and $PI^{(+)}$ annexin $V^{(+)}$ cells represented the cells in early phase of apoptosis and late phase of apoptosis (or necrosis), respectively (Fig. 3). Results suggest that apoptosis and necrosis have not been induced in U87MG and A172 cell lines after treatment with PBCA NPs.

In real-time PCR assay, to confirm correct fragments amplification, melting curve analysis and gel electrophoresis were used. The melting curve peaks were drawn at 83.6, 87.7, 83, 86.8, 88.8, 90.2 and 88°C for *TBP*, *BCL2, BCLXL*, *BCL2L12*, *BAX*, *BAD* and *BAK* genes, respectively (Fig. 4*a*). The specific length of PCR products for *TBP, BCL2, BCLXL, BCL2L12, BAX, BAD* and *BAK* genes were 96, 122, 86, 125, 101, 90 and 105 bp, respectively (Fig. 4*b*). It was understood from preliminary data that target genes and control genes expression variations would not be more than ten-fold in the treated samples. So, twofold serial dilution was used in the standard curves to prove the preciseness of real-time PCR. The efficiency of real-time PCR was 99.5, 96.1, 96.2, 99.5, 95.8, 97.7, 98.2 for *TBP*, *BCL2, BCLXL, BCL2L12, BAX, BAD* and *BAK* genes (Fig. 5). The relative standard deviations of C_T values in all the assays were <2%.

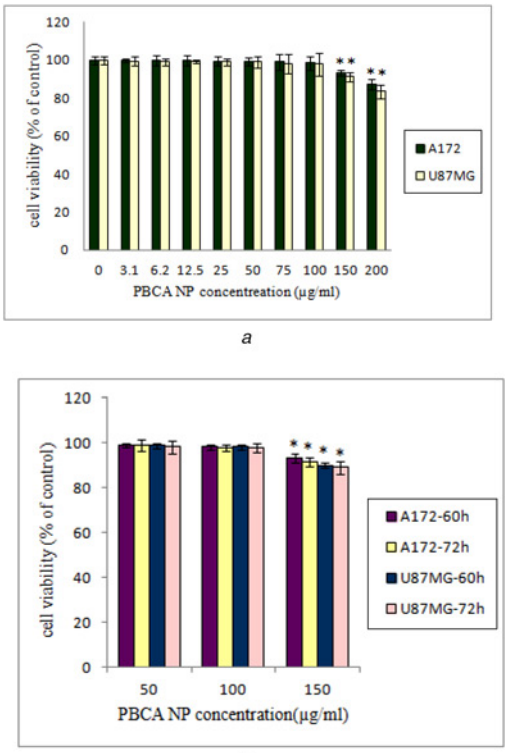

Fig. 2 *Results of cell viability assay. Data are presented as mean±SD (at least three independent repeats) of cell viability for each concentration of PBCA compared to the control. Asterisked values (related to the control) are significantly different*

a Cell viability assay results for two glioblastoma cell lines after treatment with different concentration of PBCA NPs (from 3 to 100 µg/ml) for 48 hours

b Cell viability assay results for two glioblastoma cell lines after treatment with three concentration of PBCA NPs (50, 100, 150 µg/ml) for 60 and 72 hours

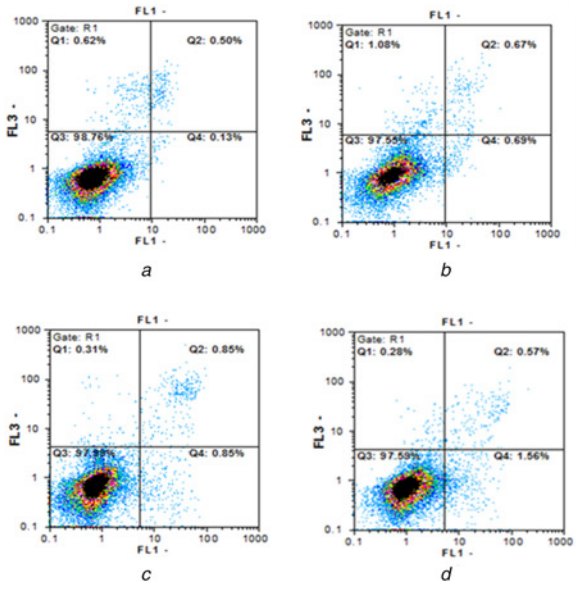

Fig. 3 *Flowcytometric evaluation of apoptosis induction in U87MG and A172 cell lines treated with PBCA NPs. Cells were stained with propidium iodide and annexin V-FITC and analysed by two-dimensional flowcytometry. The results suggest that neither apoptosis nor necrosis has been induced in U87MG and A172 cell lines after treatment with PBCA NPs. FL stands for fluorescence channels*

a A172 cell line untreated (control) sample

b A172 cell line treated with the PBCA NPs

c U87MG cell line untreated (control) sample

d U87MG cell line treated with the PBCA NPs

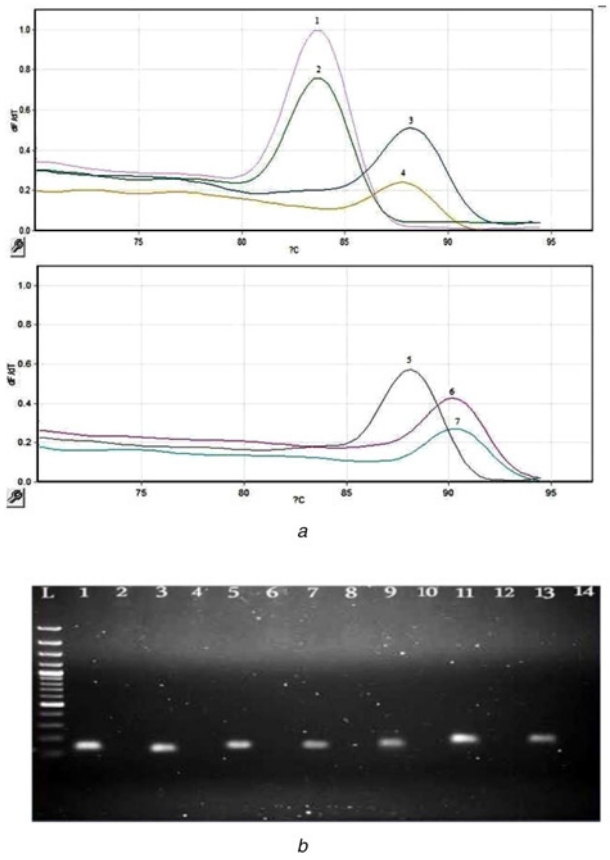

Fig. 4 *Confirmation of correct fragments amplification in Real time PCR assay using melting curve analysis and gel electrophoresis*

a Single melting peaks for *TBP* (1), *BCLXL* (2), *BAK* (3), *BCL2* (4), *BCL2L12* (5), *BAX* (6), and *BAD* (7) genes indicated the formation of specific PCR products. Fluorescence melting peaks were obtained by plotting the derivative of fluorescence over temperature (dF/dT) versus temperature (T)

b Agarose gel showing the PCR products of the real-time PCR assay: Lane L: molecular size marker (100 bp ladder), Lane1: *BCL2* PCR product, 2: NTC for *BCL2* gene, 3: *BCL2L12* PCR product, 4: NTC for *BCL2L12* gene, 5: *BAK* PCR product, 6: NTC for *BAK* gene, 7: *BAX* PCR product, 8: NTC for *BAX* gene, 9: *TBP* PCR product, 10: NTC for *TBP* gene, 11: PCR product of *BCLXL* gene, 12: NTC for *BCLXL* gene, 13: PCR product of *BAD* gene, 14: NTC for *BAD* gene

The target gene/*TBP* gene expression ratio was calculated for each gene using the formula $2^{-\Delta\Delta Ct}$. The ratios were calculated as 1.25 ($p > 0.05$), 0.12 ($p = 0.001$), 0.14 ($p = 0.001$), 0.35 ($p = 0.01$), 0.42 ($p = 0.01$), 0.24 ($p = 0.01$) for *BCL2, BCLXL, BCL2L12, BAX, BAD* and *BAK* genes in A172 cell line. The corresponding ratios were 0.79 ($p > 0.05$), 2.89 ($p = 0.01$), 0.02 ($p = 0.001$), 0.27 ($p = 0.01$), 0.10 ($p = 0.01$), 0.33 ($p = 0.01$) in U87MG cell line (Table 2).

As data showed in both A172 and U87MG cells, after treatment with non-toxic dose of PBCA NPs, the expression profile of the target genes was changed. In A172 cells, the expression of *BCLXL, BCL2L12, BAX, BAD* and *BAK* genes was decreased significantly. However, the expression of *BCL2* was slightly increased. In U87MG cells, the expression of *BCLXL* was increased while the expression of *BCL2L12*, *BAX*, *BAD* and *BAK* genes was decreased significantly. In this cell line, the expression of *BCL2* was relatively decreased which was not statistically significant.

4. Discussion: It has been accepted that the toxicity of NPs is largely determined by their physical and chemical characteristics, such as their size, shape, specific surface area, surface charge, catalytic activity, and the presence or absence of a shell and active

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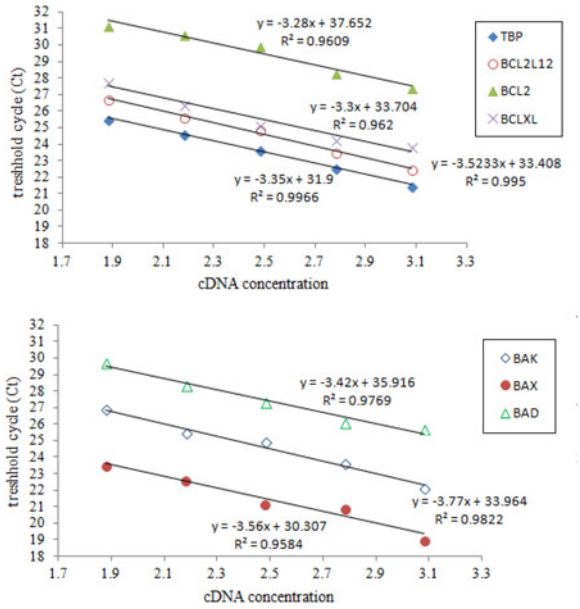

Fig. 5 *Real-time PCR assay standard curves for TBP, BCL2L12, BCL2, BCLXL, BAK, BAX and BAD genes. Standard curves were obtained, using diagrammed C_T values against logarithm of the five serial two fold dilutions cDNA concentrations*

Table 2 Relative gene expression data obtained by the real-time PCR assay for *BCL2, BCLXL, BCL2L12, BAX, BAD* and *BAK* genes in the glioblastoma cell lines treated with PBCA NPs

	Relative gene expression	
Gene	A172	U87MG
BCL2	1.25	0.79
BCLXL	0.12	2.98
BCL2L12	0.14	0.02
BAX	0.35	0.27
BAD	0.42	0.10
BAK	0.24	0.33

groups on the surface [30]. In this study, we investigated the viability and apoptosis/necrosis in U87MG and A172 glioblastoma cells after treatment with PBCA NPs. Also, the expression of six members of *BCL2* family was analysed in these cells through real-time quantitative PCR assay.

In our study, PBCA NPs treatment (from 3 to 100 µg/ml) had no significant effect on the viability of U87MG and A172 cells. Furthermore, the flowcytometric analysis confirmed that PBCA NPs treatment did not induce apoptosis and necrosis in these cells. However, gene expression analysis results showed that PBCA NPs treatment caused significant changes in the expression of some *BCL2* family members.

PBCA has attracted much attention as a drug delivery carrier. This is due to the fact that this polymer can be prepared as NPs with low toxicity and excellent biodegradability [7, 15]. In fact, it has been proved that PBCA NPs can be an effective drug delivery carrier for controlled delivery of various drugs (anticancer agents, antibiotics and peptides) [7, 15, 31]. PBCA NPs have been previously shown to entrap the curcumin effectively improving the bioavailability of curcumin [32]. Also, Paclitaxel-loaded PBCA NPs were shown to be more potent in tumour growth suppression than free Paclitaxel [14, 33]. In another study, the Thymopentin-loaded PBCA NPs have shown better pharmacodynamic effects than free drug solution [34].

Most importantly, PBCA NPs were also shown to enable the transport of a number of drugs across the BBB. For instance, PBCA NPs were successfully used for plasmids and antisense oligonucleotides delivery to brain tumours [35]. In the same way, there have been evidences that the brain targeting ability of temozolomide and doxorubicin would be enhanced when bound to PBCA NPs [7, 12, 13].

To translate the usage of nanomaterials to the clinic, their safety needs to be verified, particularly in terms of cytotoxicity and genotoxicity [20, 25]. In this study, the MTT assay and flowcytometric results showed that PBCA NPs treatment (from 3 to 100 µg/ml) for 48 h had no significant effect on the viability of U87MG and A172 glioblastoma cells. Duan *et al.* (2010) treated three kinds of hepatic cancer cells lines (HepG2, Huh7 and Bel7402 cell lines) with PBCA NPs for 12 h. They reported that the viability of the cells incubated with PBCA NPs (up to 1 mg/ml) remained about 90% relative to the control [36].

To further understand the nanotoxicity of PBCA NPs, we investigated the dynamics of the expression of the genes involved in apoptosis induction. Gene expression profiling has the potential to enhance our understanding of biological pathways in which NPs affect cells.

There are different studies which examined gene expression alteration in cell lines after treatment with toxic or non-toxic dose of various NPs [20, 37–40]. For example, it has been reported that CuO NPs treatment altered the expression of several genes in human lung epithelial A549 cells [37]. Hangata *et al.* (2010) have reported that ZnO NPs with different characteristics affect various gene expression in human lung epithelial A549 cell lines [38]. It has been also demonstrated that treatment with polymeric NP, Eudragit®, down-regulated *NCF1*, *NFKB*, and *IL1B* in human THP-1 monocytic cell lines [39]. In another study, real-time PCR array revealed that silica-coated cobalt ferrite NPs significantly increased the expression of the genes involved in stress and toxicity signal pathways in Hep3B hepatocarcinoma cell line [20].

Our results showed that the expression of *BCLXL*, *BCL2L12*, *BAX, BAD* and *BAK* genes was significantly altered in A172 and U87MG cell lines after treatment with non-toxic dose of PBCA NPs. Nevertheless, none of the glioblastoma cell lines showed apoptosis changes at cellular level. These alterations might counterbalance the effects of each other so the cumulative outcome of their contribution had no apoptosis induction effect. This is in agreement to the results of a previous study which has been shown that TiO_2 NPs are not toxic for HepG2 cells; although they can deregulate mRNA expression of DNA-damage-responsive genes (p53, p21, gadd45a and mdm2) and several antioxidant enzymes in this cell line, providing evidence that TiO_2 NPs are genotoxic [41, 42]. In the same way, Falagan-Lotsch *et al.* (2016) have reported that after Au NP treatment, very little difference was observed in cell viability of HDF cell line, but gene expression analysis showed that 25 genes related to stress and toxicity pathways were significantly altered in this cell line after treatment with Au NPs [40].

Our data also showed that these alterations are not consistent in two glioblastoma cell lines. Such differences in molecular responses between two cell lines were not unexpected as it is in agreement with other studies that showed same NP can affect different genes in different cells [39]. ZnO NPs altered the expression of 2703 genes in human monocyte-derived macrophages, 980 genes in Jurkat T cell leukemia derived cell line and only 12 genes in monocyte-derived dendritic cells [43].

5. Conclusion: In conclusion, our results showed that treatment with PBCA NPs at non-toxic dose did not induce apoptosis in two glioblastoma cell lines at cellular level but it altered expression of five members of *BCL2* family in both cell lines. However, these changes were not consistent in the two cell lines, which suggest that different molecular changes may happen in different cells in response to similar treatments. To our knowledge, this is the first

study of the effect of PBCA NPs treatment on the expression of apoptosis-related genes. Global analysis of gene expression in different cell lines might provide more precise insight of molecular effects of PBCA NPs on target cells.

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