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Hydrated C_{60} Fullerene Enhances Parthanatos and Induces Autophagy-Related Biomarkers in Glioblastoma Cell Line

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Glioblastoma, C_{60} hydrated fullerene, Autophagy, Parthanatos

Abstract: Glioblastoma is one of the most aggressive type of brain cancers, which is resistant to chemo- and radio-therapy. Nanoparticles of C_{60} fullerene derivates develop anticancer activity in various models. Therefore, we investigated the effect of water soluble hydrated C_{60} fullerene ($HyC_{60}Fn$) on the expression of PARP, Beclin1, LC3, and GFAP in human glioblastoma U373 cell. Cell viability and migration were detected by MTT and wound healing-scratch assay, respectively. The expression of PARP, Beclin1, and LC3 were analyzed by western blotting and GFAP was detected by immunocytochemistry. $HyC_{60}Fn$ in a range of doses 0.5 – 2.0 µM decreased cell viability. Furthermore, the doses of $HyC_{60}Fn$ 1.0 and 2.0 µM noticeably suppressed glioblastoma cell migration. Mechanistically, we defined that $HyC_{60}Fn$ markedly up-regulated Beclin-1 and ratio of LC3-II/LC3-I expression as autophagy markers. Furthermore, water soluble $HyC_{60}Fn$ activated cleaved PARP fragment and consequently parthanatos in glioblastoma U373 cancer cell. Present results demonstrate that $HyC_{60}Fn$ could initiate anti-tumor effect via the combination of severe autophagy flux and parthanatos in glioblastoma cells. Thus, $HyC_{60}Fn$ affects the cancer cell death machinery, at least partially, through modulating glioblastoma cells reactivity and programmed cell death. Our findings suggest that pristine hydrated C_{60} fullerene could be a promising anti-cancer therapeutics and further study is required.

Hydrated C_{60} Fullerene, Glioblastoma Hücre Hattında Parthanatosu Arttırır ve Otofaji İle İlgili Biyobelirteçleri İndükler

Anahtar Kelimeler
Glioblastoma, C_{60} hydrated fullerene, Otofaji,

Öz: Glioblastoma, kemo ve radyoterapiye karşı dirençli, en agresif beyin kanseri tiplerinden biridir. C_{60} fulleren türevi nanopartiküller, çeşitli modellerde antikanser aktivite amacı ile geliştirilmektedir. Bu nedenle, suda çözünür hydrated C_{60} fullerene'in ($HyC_{60}Fn$) insan glioblastoma U373 hücresinde PARP, Beclin1, LC3 ve GFAP ekspresyonu üzerindeki etkileri araştırılmıştır. Hücre canlılığı ve göçü, sırasıyla MTT ve yara iyileşmesi testi ile belirlendi. PARP, Beclin1 ve LC3 ekspresyonu western blot ile ve GFAP ise immünositokimya ile tespit edildi. 0.5

Parthanatos – 2.0 µM doz aralığındaki $HyC_{60}Fn$, hücre canlılığını azalttığı belirlendi. Ayrıca, $HyC_{60}Fn$ 1.0 ve 2.0 µM dozları, glioblastoma hücre göçünü belirgin şekilde bastırmıştır. Mekanizma olarak, $HyC_{60}Fn$'nin otofaji belirteçleri olarak Beclin-1'i ve LC3-II/LC3-I ekspresyon oranını belirgin şekilde yukarı regüle ettiği belirlendi. Ayrıca, suda çözünür HyC60Fn'nin PARP fragmanı ve bu durumun doğal sonuç olarak glioblastoma U373 hücrelerinde parthanatos aktive ettiği belirlendi. Mevcut sonuçlar, HyC60Fn'nin, glioblastoma hücrelerinde şiddetli otofaji akışı ve parthanatos kombinasyonu yoluyla anti-tümör etkisini başlatabildiğini göstermektedir. Bu nedenle $HyC_{60}Fn$, glioblastoma hücrelerinin reaktivitesini ve programlanmış hücre ölümünü modüle ederek en azından kısmen hücre ölüm mekanizmasını etkiler. Bulgularımız, $HyC_{60}Fn$ 'in umut verici bir kanser karşıtı terapötik olabileceğini ve bu konuda daha fazla çalışmanın gerekli olduğunu göstermektedir.

1. INTRODUCTION

Glioblastoma is one of the most aggressive types of primary brain tumors, a highly combative brain tumor in adults, and among the most lethal cancer in humans. Despite advances in the surgical and radio chemotherapy approach of glioblastoma, it is insufficient in preventing recurrence due to its important side effects and only limited effectiveness [1,2] and the mortality rate of patients remains high. Thus, more effective chemotherapy agents with fewer side effects are urgently needed. Several types of fullerene nanoparticles act as neuron protector and antioxidant [3]. On the other hand, small-size C_{60} fullerene has higher toxicity potency and leads to inhibition of DNA polymerase [4]. It should be noted that fullerene C_{60} and its derivatives may also have a toxic effect in some circumstances, whereas C_{60} can also protect the cells from the condition of oxidative damage or pathological statesThe most of reported data on fullerene bioactivity in respect to brain tissue cells were obtained with functionalized C_{60} derivates in both *in vivo* and *in vitro* studies [5–7]. C_{60} fullerene develops extremely wide range of bioactivity in dependence of a dose and the state of surface [8–10] Cytotoxic effect of several functionalized C_{60} fullerene is recognized as a function of its prooxidant effect [6, 11, 12]. Various anticancer effects of several fullerene forms were established including DNA methylation [13], cell cycle arrest [14], anti-angiogenic effect [15] and stress-induced apoptosis [16]. Besides, neuroprotective effect of pristine C_{60} fullerene on autophagy flux and apoptosis was demonstrated in rat brain stressed with hyperglycemia [17]. One of the hallmarks of malignation is the overproduction of reactive oxygen and nitrogen species (ROS/RNS) that play an importante role in cancer process [18–20]. Previous studies have emphasized that ROS/RNS, as two-faced molecules, play a dual role as a tumor promoting agent depending on the intracellular level or tumor-suppressing due to their implication in triggering cell death [20–22]. However, redox abnormality is not specific for tumor cells. There is a rising evidence that prevaling number, if not all, of various cellular disturbances are accompanied by increased free radicals generation. Despite wide studied pro-oxidant effects of functionalized fullerene, pristine C_{60} fullerene could inhibit tumor progress via modulation of regulatory pathways and gene expression [17, 23]. Glioblastoma cells progress depends on multiple mutations and abnormal regulation of cell death pathways, including resistance to programmed cell death initiation [24]. Programmed cell death, particularly apoptosis and autophagy, plays a fundamental role in the cellular strategy to maintain the balance in surviving and elimination of damaged cell [25]. However, the modulation of programmed cell death is promising therapeutic development to suppress cancer growth too, including the autophagy lysosomal pathway in gliomas [26]. Autophagy is described as a self-cannibalism that is a highly conserved dynamic cellular process occurring as a cellular response to starvation or pathogen infection that degrades macromolecules or organelles [27]. The formation of the self-cannibalism mechanism is initiated with encapsulating of macromolecule or organelles into double-membrane intracellular vesicles and then fused with lysosomes to be recycled [28, 29]. Previous studies have indicated that autophagy, which is characterized by the presence of auto-phagosome, plays a dual role as a lysosomal degradation pathway and autophagy depend-cell death, inhibiting cancer growth depending on the intracellular stress of cells [29, 30]. Previous studies have emphasized that C_{60} fullerene, as two-faced molecules, could induce accumulation of ROS [31] or in contrast, serves reduce oxidative damage and ROS level [32]. Autophagy-related proteins (Atg), Beclin-1, and microtubule-associated protein 1A/1B-light chain 3 (LC3) are central regulatory proteins in autophagy. Beclin-1 is an important regulator involved in the initiation of autophagy [33]. In the autophagy pathway, Beclin-1 and LC3 which is called Atg8, have non-negligible function in the autophagosome membrane. [29, 34]. Besides, autophagy-dependent cell death, non-apoptotic cell death signaling pathways including parthanatos have recently gained great interest [35]. Parthanatos, other cell death pathway, is a poly(ADP-ribose) synthetase 1 (PARP1)- dependent cell death that initiated by overactivation of PARP1 accompanied by depletion of NAD and ATP [35, 36]. Parthanatos is tightly associated with various macromolecular damages and mitochondrial dysfunction as well as other types of programmed cell death [35]. Despite many factors contribute to parthanatos, it does not require caspases participation [36]. Overexpression of PARP1, which in turn leads to a number of biological consequences, induces apoptosis-inducing factor (AIF), release into the cytoplasm, and then is translocated into the nucleus where it triggers chromatin condensation and DNA fragmentation [36, 37]. The effect of fullerene on parthanatos initiation remains unknown. Besides, there are limited data in respect with the suppression of glioblastoma progress by fullerene exposure [7, 38]. GFAP is a glial specific intermediate filaments protein

and its overexpression is the main marker of astrogliosis [39]. Furthermore, there was reported that expression of GFAP isoforms involved in glial cell motility and proliferation where dynamic rearrangement of intermediate filaments network is accompanied by the modulation of focal adhesion [40]. However, the role of glial intermediate filaments in cell motility remains enigmatic. Thus, the aim of the present study was to elucidate the effect of C_{60} fullerene on Beclin1, LC3 and PARP expression, and migratory activity in human glioblastoma U373 cell line.

2. MATERIAL AND METHOD

2.1. Cell Line and Culture Condition

The human glioblastoma cell line (U373 MG) was purchased from the American Type Culture Collection (ATCC, American Type Culture Collection; Rockville, MD, USA). The cells were grown in culture dishes in DMEM medium supplemented with 10% fetal bovine serum. The medium was also supplemented with 64 µg/ml penicillin (cat. No. A1837.0010) + 0.1 mg/ml streptomycin (cat. No. A1852.0025; both VWR, USA) antibiotic solution. The cells were incubated in 5% CO_2 at 37 °C and 80–90% confluence, cells were carefully removed with trypsin/EDTA and washed with phosphate buffered saline (PBS).

2.2. Determination of Cell Viability

The cell viability was determined as previously described [41] by the MTT assay. Briefly, to determine the C_{60} fullerene inhibitory potency on growth, 6,000 cells were seeded into each well of 96-multiwell cell culture plates in culture medium containing 10% FBS. The cells were treated with various concentrations of C_{60} (0.5, 1.0 and 2.0 µM) allowed to grow for 24 h. After that, 10 µl of MTT labelling reagent, 3-(4, 5- dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide (MTT) stock solution (0.5 mg/ml) was added to each well and incubated for 4 h at far from light. The purple-coloured formazan pellets were dissolved in 100 µl DMSO, incubated for 15 min and read using a microplate reader (Molecular devices LLC, USA) at 550-600 nm (the reference wavelength 650 nm).

2.3. Migration Test

The day before treatment, U373 cells (5×10^4 cells/dish) were seeded into 12 wells plate for attachment. A mechanical scratch was made with the help of a sterile 200 µl pipette tip, and the plates were then washed with PBS to remove detached cells. The cells were treated with 1 and 2 µM of C_{60} for 24 h. The wells were photographed at different time points. Cell migration pictures were monitored using an invert microscope (Olympus, CKX41, Tokyo, Japan) at 40× magnification.

2.4. Western Blotting

U373 cells (1×10 6 cells/dish) were maintained on a 10-cm dish with DMEM medium containing 10% FBS for 24 h and were incubated with 24 h of C_{60} fullerene for 0, 0.5, 1, and 2 µM. After treatment, cells were collected by scratching without trypsinization and suspended in a fresh lysis buffer (RIPA) plus 1 mM PMSF (phenyl methane sulfonyl fluoride) and a proteinase and phosphatase inhibitor cocktail as described previously [41]. Protein concentrations were measured and re-suspended in a loading buffer. A total 30 µg from each sample was electrophoresed by 10 or 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride membranes (PVDF, Millipore, Germany/USA; cat. No. IPVH00010). 5% non-fat milk was used for blocking the membrane, which was then incubated with primary antibodies (Beclin1, LC3 and PARP diluted 1:1,000, β-actin – 1:3,000, Santa Cruz, CA, USA) at 4◦ C overnight. The membranes were then incubated with secondary antibody (anti-mouse IgG-HRP and anti-rabbit IgG-HRP-1:5,000, Advansta, California, USA) that conjugated with horseradish peroxidase for 1 h at room temperature with gentle agitation. The signal was developed by an enhanced chemiluminescence method with the use of X-ray films (Carestream, USA). Densitometry analysis was performed using the ImageJ software (USA) and normalized against its respective loading control.

2.5. Immunofluorescence

GFAP immunocytochemistry (ICC) was performed in U373 cells according to the immunofluorescence assay protocol as described earlier [42]. Briefly, fixed and permeabilized cells were washed with cold PBS and then cells were blocked with 5% BSA by 60 min at 37°C. Cells were then incubated with primary anti-GFAP antibody (1:200 dilutions in PBS-Tween, Santa Cruz, CA, USA) overnight at 4°C. Cells were washed with PBS-Tween for 15 min. Secondary anti-rabbit antibodies (Sigma-Aldrich, USA) conjugated with green fluorophore fluorescein isothiocyanate (FITC) in 1:400 dilution were added and incubated for 60 min at 37 °C. After that, the cells were washed again three times with PBS-Tween for 15 min, nuclei were counterstained with blue fluorescent dye Hoechst-33342 (1 µg/ml). Fluorescent images captured using the Zeiss LSM510 Meta confocal microscope were converted to gray-scale and normalized to background staining. Specific signal of GFAP immunofluorescence from the at least nine regions of interest (ROI) of each group of cells was evaluated using the ITCN plugin for ImageJ (https://imagej.nih.gov/ij/). Quantification of GFAP-positive U373 cells was measured as the percentage of area occupied by fluorescent-labeling in each ROI and then correlated with the number of nuclei located in each ROI. GFAP-positive signal intensities were expressed as % from the control level.

2.6. Statistical Analyses

Cell viability, western blot, and values of GFAP immunofluorescence results were evaluated by analysis in Graph Pad Prism 5.01 program. The result were analysed by one-way ANOVA followed by post-hoc Tukey test and the analysis were carried out for three replications. P level less than 0.05 was considered statistically significant.

3. RESULTS

3.1.Effects of C_{60} Fullerene on U373 Cell Viability

First, we examined the effect of C_{60} fullerene on cell viability in U373 cell line with different concentration of C_{60} fullerenes (0, 0.1, 0.25, 0.5, 1.0 and 2.0 µM) for 24 h. The cell viability was assessed by MTT assay measurements. As shown in Figure 1, after 24 h treatment of C_{60} fullerene, it was observed that C_{60} fullerene inhibited cell growth. The U373 cell viability was almost unchanged at a low dose as compared with the control group, whereas, it was reduced at the high dose of 1 and 2 µM, indicating the most effective concentration.

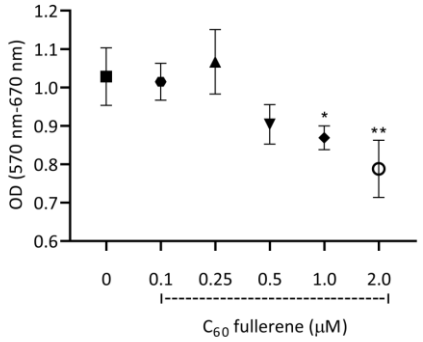


Figure 1. Effects of C_{60} fullerene on cell viability in U373 cell line. Concentrations of C_{60} fullerene, µM, are shown below the diagrams. *P < 0.05 and **P <0.01 vs. the control group.

3.2.Effects of C_{60} Fullerene on U373 Cell Migration

Next, we explored whether C_{60} could suppress cell migration in U373 cell line. The U373 migrative activity was detected as one of the features of tumorigenicity as well as invasive and metastasis index. Present wound healing assay results have shown that the doses of C_{60} 1.0 and 2.0 µM were able to inhibit the migration of U373 glioblastoma cells (Figure 2).

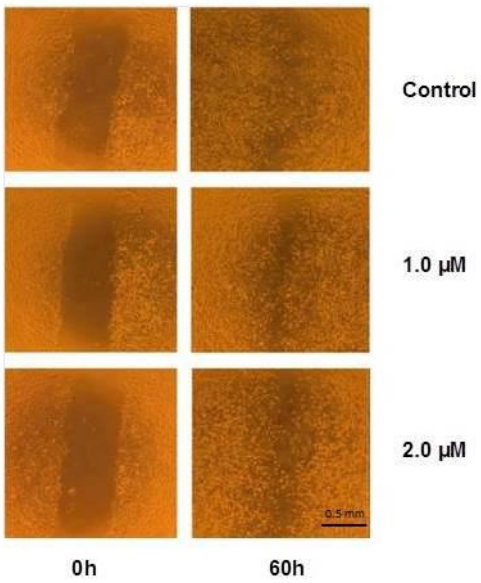
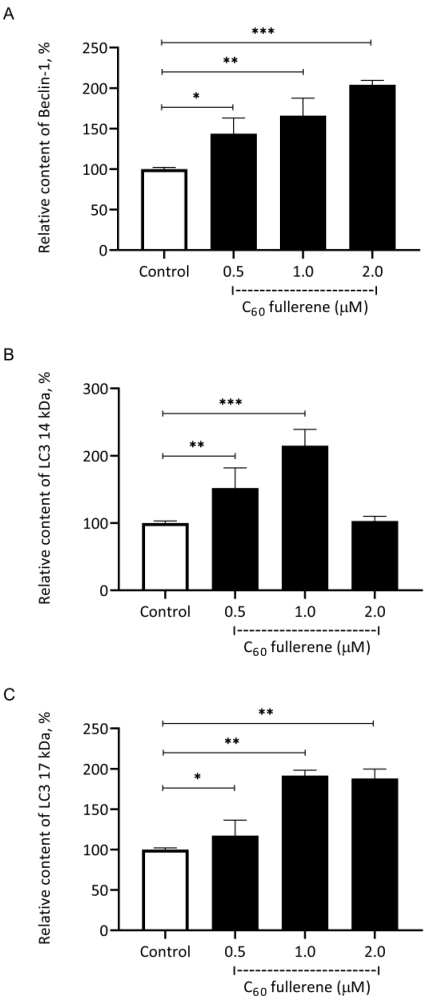


Figure 2. Effects of C_{60} fullerene on the cell migration in U373 cell line. Wound healing-scratch assays were performed to evaluate cell migration. The images were acquired at 0 h and 60 h.

3.3.Effects of C_{60} Fullerene on Beclin1, LC3 and PARP Expression

Given the above results, we further examined the protein expression of Beclin1, LC3 and PARP. It is reported that various anti-cancer agents increase ROS production and induce the activation of autophagy-mediated cell death in malignant glioma [43, 44]. Western blot results demonstrated that C_{60} treatment increased Beclin 1 protein expression in U373 cell line (Figure 3). Moreover, as quantified accordingly western blot results, while the cells treated with 2 µM markedly upregulated the levels of Beclin1 compared to the control group, treatment with 0.5 or 1.0 µM C_{60} did not reach statistical significance. Furthermore, LC3-II significantly increased in U373 cells subjected to 1.0 µM C_{60} treatment compared to at low dose and the untreated cells. To further confirm the roles of C_{60} treatment on U373 cells, western blotting analysis was carried out after treating with C_{60} treatment for 24 h. PARP protein expression was significantly upregulated in the high dose treatment group when compared to the control group. Nevertheless, treatment with 0.5 µM C_{60} did not reach statistical significance.



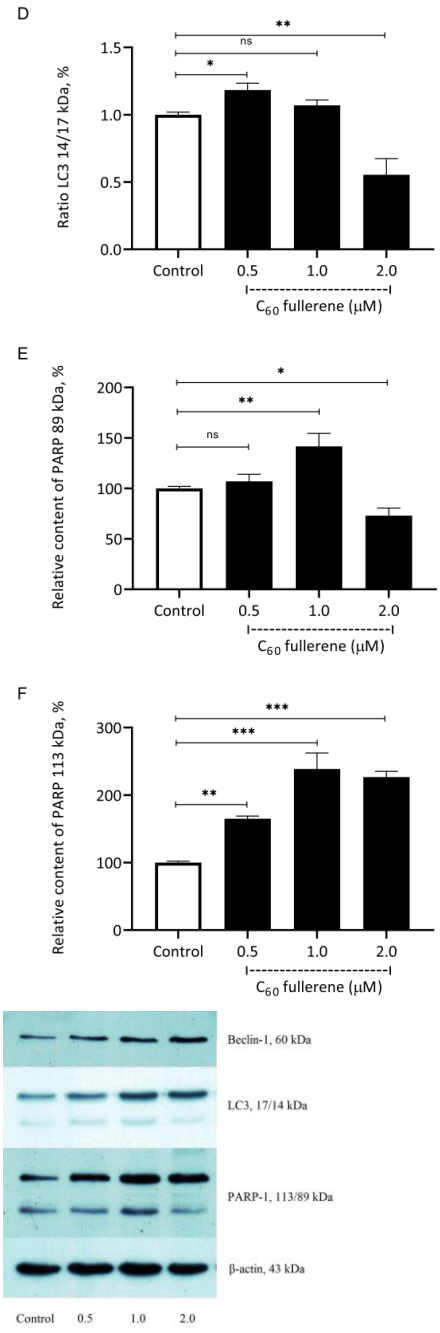


Figure 3. Effects of C_{60} fullerene on Beclin1, LC3 and PARP expression in U373 cell line.Western blot results of Beclin1 (A), LC3-14kDa (B), LC3-17kDa (C), LC3-14/17kDa (D), PARP-89kDa (E), PARP-113kDa (F) proteins in U373 cells treated with C_{60}. The results were normalized using protein expression rates of β-actin. Compared with the control group, *: P<0.05, **: P<0.01, *** P<0.001.

3.4.Effects of C_{60} Fullerene on GFAP

To further establish the effect of C_{60} fullerenes on U373 glioma cell reactivity, GFAP expression was investigated by immunocytochemistry as a marker of glial reactivity. As shown by immunocytochemistry imaging, C_{60} fullerene suppressed GFAP expression in U373 cells as compared to control non-treated cells (Figure 4.). The obtained result means that C_{60} fullerene is able to modulate important determinants of tumor invasiveness such as cell migration and adhesion by modulating expression of intermediate filament protein of astrocytic cytoskeleton, and thus may diminish tumor infiltration.

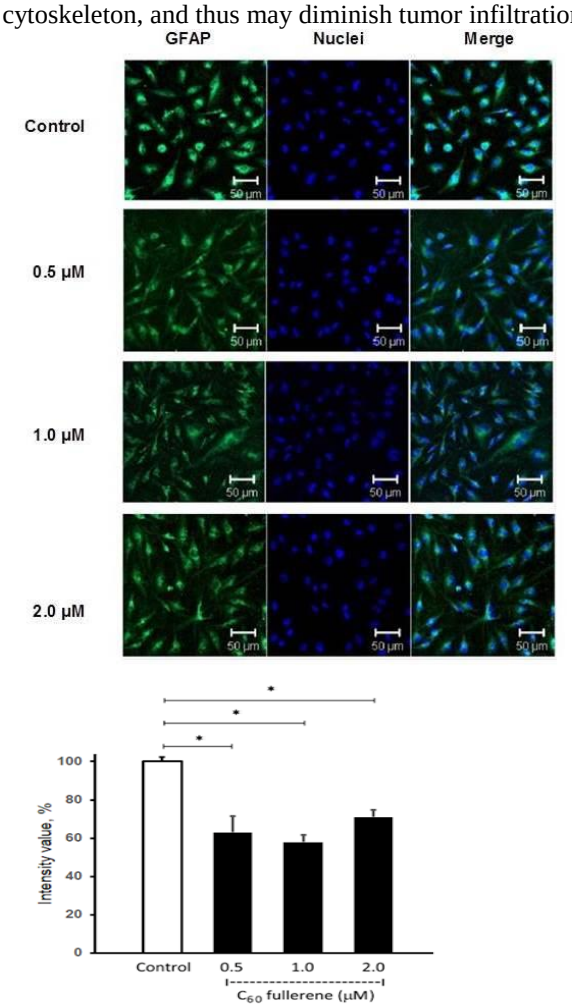


Figure 4. Immunocytochemical staining of GFAP in C_{60} fullerene treated U373 cells. Concentrations of C_{60} fullerene, μM, are shown on the diagrams. *P < 0.05 vs. the control group.

4. DISCUSSION AND CONCLUSION

Biomedical potential to apply C_{60} fullerene as an anticancer tool is based on its capability to initiate the switching programmed cell death to apoptosis, necrosis or both of them. Well studied manner to initiate apoptosis is ROS-dependent pathways which is reported as the most prospective to suppress tumor growth [12]. Contrary, the effects of C_{60} fullerene via modulating programmed cell death remain unknown. However, research data on the impact of hydrated water-soluble C_{60} fullerene in GBM cells are absent in literature. There is extremely limited number of the reports in regards with pristine water-soluble C_{60} fullerene types and water suspensions in glioma cells where cytotoxic effect was detected in a wide range of concentrations [5, 38]. The results obtained in our study showed that U373 cells exposure to $HyC_{60}Fn$ exerts cytotoxity as well as the modulation of autophagy flux in a dose dependent manner. Thus, our results are consistent with literature data presented in respect to various water soluble C_{60} fullerene types [5, 7, 38] On other hand, cytotoxic effect of water soluble $HyC_{60}Fn$ in glioblastoma cells is presented for the first time. It deserves to be mentioned that meaningful cytotoxicity of C_{60} is observed

in a large concentrations range, but as a rule these doses most high then 1 μM [38, 45, 46]. The measurement of autophagy markers Beclin1 and LC3 in our study have shown a significant increase in both of them. Especially, high $HyC_{60}Fn$ doses 1-2 μM have induced significant up-regulation in Beclin1 and LC3 expression (Figure 2 A and B). Thus, $HyC_{60}Fn$ could regulate autophagy flux in U373GM cells. There was demonstrated that mild autophagy enhancement can maintain cell viability in a course of starvation or exposure to other detrimental factors [47]. Despite of this phenomenon, severe autophagy flux can induce cell death via total cleaving of macromolecules [48]. Data presented in our study indicate that $HyC_{60}Fn$ doses 1- 2 μM can initiate extensive autophagy flux in U373GM cells and limit cell viabilty at least partialy by this type of programmed cell death. Beclin1 cannot inhibit anti-apoptotic function of Bcl-2, when it absorbed on the mitochondrial membrane surface. Despite of this fact, anti-apoptotic Bcl-2 proteins Bcl-2 or Bcl-XL that forms the complex with Beclin1 can reduce its pro-autophagy activity [49]. Recently, there was demonstrated that C_{60} fullerene can modulate autophagy flux via Bcl-2/Beclin-1 reciprocal expression in rat brain [17]. Thus, obtained results are adjust with the effect of pristine C60 fullerene on Bcl-2/Beclin-1 pathway in glial tumor cells. Taking into account that malignation is tightly linked to impaired mitochondrial function and the state of mitochondrial membrane affect on interaction of autophagy-regulating proteins, including Bcl-2 and Beclin1, observed in study C60 fullerene effect on glioblastoma suppression could be mediated with modulation of mitochondrial functions [50, 51]. Harhaji and coauthors demonstrated the role of autophagy in C_{60} fullerene-dependent cell death where inhibition of acidification of intracellular vesicles initiated cell death in glioma exposed to relatively low C_{60} fullerene dose [38]. Actualy, the observed modulation of Beclin1 and LC3 expression by pristine C_{60} fullerene can reflect only one of multifaceted effects of tumor suppression in the present study. PARP overexpression can lead to cell death pathway called parthanatos [52]. The impact of PARP modulation in glioblastoma cells was reported in several studies [53, 54]. However, there are no data on the modulation of parthanatos flux with pristine fullerene nanoparticles. Our results on the up-regulation of PARP in U373GM cells provide evidence that $HyC_{60}Fn$ could up-regulate other PARP-dependent cell death pathway called parthanatos. Observed increase in both PARP expression and cleaved PARP fragments let us to presume $HyC_{60}Fn$-induced parthanatos in gliobastoma cells. To the best of our knowledge, the data on C_{60}-induced parthanatos in gliobastoma cells are presented firstly. During the last decades, several methods were proposed to produce water soluble C_{60} nanoparticles based on solvent-exchange manner, water suspension and chemical functionalization with various groups including hydroxilation and carbonylation [5, 55]. However, prevaling number of these methods generate extremely various nanoparticles with different biology activity without any principle standards. Similar great vary of cytoprotective and cyto-toxic effects C_{60} nanomaterials are presented in literature. Therefore, the distinct effect of C_{60} fullerene solubilized with various manner could be explaned individual features of carbon cage surface. The state of the fullerene surface is a critic for exhibiting both cytoprotective and antitumor effects. Several water-soluble C_{60} fullerene derivatives were demonstrated as the prospective agents to inhibit glioblastoma in vitro [5]. On other hand, various cancer cell types have a unique complex of mutations and are susceptible to specific manner of anti-cancer strategy. Therefore, multiple features of various water soluble C_{60} fullerene types are requested to be identified in brain tumor cells. In respect with neural tissue cells, the effect of C_{60} fullerene has been described in several reports including neuroprotection against amyloid peptide [56], hypoxic insult [57] and glutamate toxicity via modulating cytoskeleton-associated proteins [57]. Hydrated C_{60} fullerene was detected to be capable of reducing serum homocysteine level and TRPM2 gene expression in vivo [23]. Enhancing effect of the water-soluble C_{60} fullerene derivatives has been shown on the neurite outgrowth of NGF-stimulated cultured PC12 cells in vitro [58]. Dugan and coauthors reported that polyhydroxylated C_{60} derivatives possess the potential to reduce excitotoxic and apoptotic injuries in cortical cell cultures due to its anti-oxidant features [55]. Thus, pristine C_{60} fullerene is capable of modulating various pathways in neural tissue cells that could recognized as an argument to apply it as anti-glioma agent. There are numerous contraversial reports on anti-oxidant and/or pro-oxidant activity of pristine C_{60} fullerene in both normal and malignated cells [5, 7]. However, normal cells in common have much higher resistance to pro-oxidant C_{60} fullerene effect than their cancerous counterparts [10, 38, 59]. Furthermore, pristine fullerene is recognized as nontoxic compound up to 2 mg/kg [60] The most important property of pristine of the C_{60} fullerene is low toxicity in respect with normal cells. The absent of abnormalities in the rats were observed in both acute and chronic exposure to 2,000 mg/kg and 1,000 mg/kg, respectively [60, 61]. Besides, no histopathological changes were detected in liver, kidney and spleen in the end of 28 days administration period as well as the accumulation of fullerene C_{60} were not detected in all of aforementioned organs [61]. Contrary, there are several reports on neurotoxicity and cytotoxicity of various C_{60} fullerene types. For instance, pristine unfunctionalized C60 fullerene in high concentration (100 μg/mL) have been detected to be able to induce cytotoxicity in both undifferentiated and differentiated by growth factor exposure PC-12 cells [8]. Comparative analyses of cytotoxicity among water-soluble fullerene species, nano-C_{60}, a fullerene aggregate, have showed that C_{60} colloidal suspension is potent to induce various cellular damages [62]. However, there were no detected DNA and mitochondrial disturbances in fibroblasts, liver carcinoma cells (HepG2), and astrocytes in aforementioned study. Anti-cancer effect of C_{60} fullerene water suspension was demonstrated in gioma cells where fullerene suppressed tumor cell proliferation [38]. Astrocytes transition into reactive state is a process that is characterized by morphological and biochemical changes by loosing the original properties of cells and increased vimentin expression as one of the intermediate filament protein [63]. The results of ICC assay obtained in our study demonsrated the mild suppression of GFAP staining

in glioma cells exposed to all applied doses of C_{60} fullerene. Taking into account the present results and the fact that GFAP is involved in astrocyte reactivity, C_{60} fullerene exposure could inhibit the rearrangement of glioma cytoskeleton via reprograming parthanatos and autophagy fluxes. Firstly, hydrated C_{60} fullerene has been shown to markedly ameliorate astroglial reactivity and modulate GFAP expression in vivo in brain tissue of rats chronically exposed to ethyl alcohol [64]. The down-regulation of GFAP caused by C_{60} treatment is agreed with early explored effect of pristine fullerene on glial intermediate filaments in brain and retina of rats with experimental diabetes mellitus [65, 66]. The current study provides the first evidence that the C_{60} fullerene can regulate expression of intermediate filament protein of the astrocytic cytoskeleton that is accompanied by enhanced parthantos and up-regulation in Beclin1 and LC3 autophagic markers in the glioma U373 cells. Cell migration activity plays a key role in the invasion and metastasis of GBM. We used cell migration/wound healing test to determine the effect of C_{60} fullerene on GBM cells migration activity. The present results (Fig. 2) demonstrated that C_{60} fullerene doses 1 and 2 µM inhibited migration capability of GBM during 60 h-period of observation. Obtained data indicate that these doses of hydrated C_{60} fullerene can suppress GBM migration through massive programmed cell death and reactivity of GBM cells. The various cellular disturbances were observed glioma cells exposed to high dose (≥1 µg/ml) and lower concentration (0.25 µg/ml) including stimulation of extracellular signalregulated kinase (ERK), growth of acidified intracytoplasmic vesicles indicative of autophagy and ROS-mediated necrotic cell damage. Furthermore, the exposure to high dose predominantly induced cell death by necroic pathway. On the other hand, the low dose of C_{60} fullerene water suspension had no effect on glioma necrosis, but this low C_{60} concetration could provoke the increment in cancer cells accumulation in G2/M phase that is an index of the cell cycle arrest. The authors showed that cytostatic effect of low-dose C_{60} was only less significant in primary astrocytes then in transformed glial cell, but it has no absent [38]. Distinct cytotoxic mechanisms were observed in respect with anticancer effect of pristine C_{60} fullerene and water-soluble polyhydroxylated fullerene. Despite necrotic effect of pristine C_{60} nanocrystals, hydroxylated fullerene C_{60} may induce various apoptotic events, including DNA fragmentation, ROS-independent cell death with characteristics of apoptosis and loss of the specific shape of cellular membrane [6]. Therefore, C_{60} nanoparticles exhibit extremely various bioactivity depending on the dose, the manners for solubilization and the rate of hydratation of C_{60} nanoparticles, which are critic for cytotoxic and cytoprotective features of C_{60} fullerene and its derivates. Correlation between autophagy flux and parthanatos observed in our study uncovers the part of antiglioma effect of pristine hydrated C_{60} fullerene. Taking into account that hydrated fullerene is able to interact with proteins [67], we can presume hypotetic mechanism in which direct effect of C_{60} on cell death machinery could be involved, at least partialy, in the modulation of autophagy and parthanatos. Presented results demonstrate dose-dependent cytotoxicity of water-soluble $HyC_{60}Fn$ in U373GM cells. Furthermore, the doses of 1.0 and 2.0 µM $HyC_{60}Fn$ could initiate anti-tumor effect via the combination of severe autophagy flux and parthanatos in glioblastoma cells. Therefore, pristine hydrated C_{60} fullerene displays potent anti-cancer features and further study is required.

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