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

Aryan M. FARAJ\textsuperscript{1,2}, Victor S. NEDZVETSKY\textsuperscript{1,3}, Artem A. TYKHOMYROV\textsuperscript{4}, Giyasettin BAYDAS\textsuperscript{5}, Abdullah ASLAN\textsuperscript{6}, Can Ali AGCA\textsuperscript{7}

\textsuperscript{1}Department of Molecular Biology and Genetics, Bingol University, 12000, Bingöl, Türkiye
\textsuperscript{2}Department of Medical Laboratory Science, Sulaimani Polytechnic University, Sulaymaniyyah, Iraq
\textsuperscript{3}Department of Physiology, Biochemistry of Animals and Lab Diagnostics, Dnipro State Agrarian and Economic University, 49600, Dnipro, Ukraine
\textsuperscript{4}Department of Enzyme Chemistry and Biochemistry, Palladin Institute of Biochemistry of the National Academy of Sciences of Ukraine, Kyiv, Ukraine
\textsuperscript{5}Department of Enzyme Chemistry and Biochemistry, Altinbash University, 34218 Istanbul, Türkiye
\textsuperscript{6}Faculty of Science, Department of Biology-Molecular Biology and Genetics Program, Firat University, Elazig, Türkiye

Aryan M. FARAJ ORCID No: 0000-0002-7229-3717
Victor S. NEDZVETSKY ORCID No: 0000-0001-7352-441X
Artem A. TYKHOMYROV ORCID No: 0000-0003-2063-4636
Giyasettin BAYDAS ORCID No: 0000-0002-9206-3177
Abdullah ASLAN ORCID No: 0000-0002-6243-4221
Can Ali AGCA ORCID No: 0000-0002-0244-3767

\textsuperscript{*Corresponding author: caagca@bingol.edu.tr}

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Abstract: Glioblastoma is one of the most aggressive type of brain cancers, which is resistant to chemo- and radio-therapy. Nanoparticles of C\textsubscript{60} fullerene derives develop anticancer activity in various models. Therefore, we investigated the effect of water soluble hydrated C\textsubscript{60} fullerene (HyC\textsubscript{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\textsubscript{60}Fn in a range of doses 0.5 – 2.0 \textmu M decreased cell viability. Furthermore, the doses of HyC\textsubscript{60}Fn 1.0 and 2.0 \textmu M noticeably suppressed glioblastoma cell migration. Mechanistically, we defined that HyC\textsubscript{60}Fn markedly up-regulated Beclin-1 and ratio of LC3-II/LC3-I expression as autophagy markers. Furthermore, water soluble HyC\textsubscript{60}Fn activated cleaved PARP fragment and consequently parthanatos in glioblastoma U373 cancer cell. Present results demonstrate that HyC\textsubscript{60}Fn could initiate anti-tumor effect via the combination of severe autophagy flux and parthanatos in glioblastoma cells. Thus, HyC\textsubscript{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\textsubscript{60} fulleren could be a promising anti-cancer therapeutics and further study is required.

Keywords
Glioblastoma, C\textsubscript{60} hydrated fullerene, Autophagy, Parthanatos

Hydrated C\textsubscript{60} Fullerene, Glioblastoma Hücresi Hattında Parthanatosu Arttırmır ve Otofaji İle İlgili Biyobilirleteçleri İndükler

Öz: Glioblastoma, kemo ve radyoterapiyeye karşı dirençli, en agresif beyin kanseri tiplerinden biridir. C\textsubscript{60} fulleren türevi nanopartiküller, çeşitli modelde antikanser aktivite amacı ile geliştirilmektedir. Bu nedenle, suda çözünür hydrated C\textsubscript{60} fulleren'in (HyC\textsubscript{60}Fn) insan glioblastoma U373 hücrelerinde PARP, Beclin1, LC3 ve GFAP ekspresyonu üzerindeki etkileri araştırılmıştır. Hücresi canlulığı 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
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 states. The 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]. Cytoxic 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 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 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]. 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and its overexpression is the main marker of astrogliosis [39]. Furthermore, there was reported that expression of GFAP isosforms 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 C60 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% CO2 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 C60 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 C60 (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 C60 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 C60 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 fluorochrome 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 C₆₀ Fullerene on U373 Cell Viability

First, we examined the effect of C₆₀ fullerene on cell viability in U373 cell line with different concentration of C₆₀ 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 C₆₀ fullerene, it was observed that C₆₀ 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](image1.png)

**Figure 1.** Effects of C₆₀ fullerene on cell viability in U373 cell line. Concentrations of C₆₀ fullerene, μM, are shown below the diagrams. *P < 0.05 and **P <0.01 vs. the control group.

3.2. Effects of C₆₀ Fullerene on U373 Cell Migration

Next, we explored whether C₆₀ 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 C₆₀ 1.0 and 2.0 μM were able to inhibit the migration of U373 glioblastoma cells (Figure 2).

![Figure 2](image2.png)

**Figure 2.** Effects of C₆₀ 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 C₆₀ 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 C₆₀ 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 C₆₀ did not reach statistical significance. Furthermore, LC3-II significantly increased in U373 cells subjected to 1.0 μM C₆₀ treatment compared to at low dose and the untreated cells. To further confirm the roles of C₆₀ treatment on U373 cells, western blotting analysis was carried out after treating with C₆₀ 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 C₆₀ did not reach statistical significance.

![Figure 3](image3.png)
Figure 3. Effects of C₆₀ 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 C₆₀. 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 C₆₀ Fullerene on GFAP

To further establish the effect of C₆₀ fullerene on U373 glioma cell reactivity, GFAP expression was investigated by immunocytochemistry as a marker of glial reactivity. As shown by immunocytochemistry imaging, C₆₀ fullerene suppressed GFAP expression in U373 cells as compared to control non-treated cells (Figure 4.). The obtained result means that C₆₀ 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 C₆₀ fullerene treated U373 cells. Concentrations of C₆₀ fullerene, μM, are shown on the diagrams. *P < 0.05 vs. the control group.

4. DISCUSSION AND CONCLUSION

Biomedical potential to apply C₆₀ 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 C₆₀ fullerene via modulating programmed cell death remain unknown. However, research data on the impact of hydrated water-soluble C₆₀ fullerene in GBM cells are absent in literature. There is extremely limited number of the reports in regards with pristine watersoluble C₆₀ 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 HyC₆₀Fn exerts cytotoxicity 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 C₆₀ fullerene types [5, 7, 38] On other hand, cytotoxic effect of water soluble HyC₆₀Fn in glioblastoma cells is presented for the first time. It deserves to be mentioned that meaningful cytotoxicity of C₆₀ 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\textsubscript{60}Fn doses 1-2 µM have induced significant up-regulation in Beclin1 and LC3 expression (Figure 2 A and B). Thus, HyC\textsubscript{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\textsubscript{60}Fn doses 1-2 µM can initiate extensive autophagy flux in U373GM cells and limit cell viability at least partially 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-autophagic activity [49]. Recently, there was demonstrated that C\textsubscript{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 C\textsubscript{60} 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 C\textsubscript{60} 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\textsubscript{60} fullerene-dependent cell death where inhibition of acidification of intracellular vesicles initiated cell death in glioma exposed to relatively low C\textsubscript{60} fullerene dose [38]. Actually, the observed modulation of Beclin1 and LC3 expression by pristine C\textsubscript{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\textsubscript{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\textsubscript{60}Fn-induced parthanatos in glioblastoma cells. To the best of our knowledge, the data on C\textsubscript{60}-induced parthanatos in glioblastoma cells are presented firstly. During the last decades, several methods were proposed to produce water soluble C\textsubscript{60} nanoparticles based on solvent-exchange manner, water suspension and chemical functionalization with various groups including hydroxilation and carbonylation [5, 55]. However, prevailing number of these methods generate extremely various nanoparticles with different biology activity without any principle standards. Similar great vary of cytoprotective and cytoxic effects C\textsubscript{60} nanomaterials are presented in literature. Therefore, the distinct effect of C\textsubscript{60} fullerene solubilized with various manner could be explained individual features of carbon cage surface. The state of the fullerene surface is a critica for exhibiting both cytoprotective and antitumor effects. Several water-soluble C\textsubscript{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\textsubscript{60} fullerene types are requested to be identified in brain tumor cells. In respect with neural tissue cells, the effect of C\textsubscript{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\textsubscript{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\textsubscript{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\textsubscript{60} derivatives possess the potential to reduce excitotoxic and apoptotic injuries in cortical cell cultures due to its antioxidant features [55]. Thus, pristine C\textsubscript{60} fullerene is capable ofmodulating various pathways in neural tissue cells that could recognized as an argument to apply it as anti-glioma agent. There are numerous controversial reports on anti-oxidant and/or pro-oxidant activity of pristine C\textsubscript{60} fullerene in both normal and malignant cells [5, 7]. However, normal cells in common have much higher resistance to pro-oxidant C\textsubscript{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\textsubscript{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\textsubscript{60} were not detected in all of aforementioned organs [61]. Contrary, there are several reports on neurotoxicity and cytotoxicity of various C\textsubscript{60} fullerene types. For instance, pristine unfunctionalized C\textsubscript{60} 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\textsubscript{60}, a fullerene aggregate, have showed that C\textsubscript{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\textsubscript{60} fullerene water suspension was demonstrated in glioma 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 demonstrated the mild suppression of GFAP staining.
in glioma cells exposed to all applied doses of C\textsubscript{60} fullerene. Taking into account the present results and the fact that GFAP is involved in astrocyte reactivity, C\textsubscript{60} fullerene exposure could inhibit the rearrangement of glioma cytoskeleton via reprogramming paranhastos and autophagy fluxes. Firstly, hydrated C\textsubscript{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\textsubscript{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\textsubscript{60} fullerene can regulate expression of intermediate filament protein of the astrocytic cytoskeleton that is accompanied by enhanced paranhastos 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\textsubscript{60} fullerene on GBM cells migration activity. The present results (Fig. 2) demonstrated that C\textsubscript{60} fullerene doses 1 and 2 \(\mu\text{M}\) inhibited migration capability of GBM during 60 h-period of observation. Obtained data indicate that these doses of hydrated C\textsubscript{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 (\(\geq 1\ \mu\text{g/ml}\)) and lower concentration (0.25 \(\mu\text{g/ml}\)) 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\textsubscript{60} fullerene water suspension had no effect on glioma necrosis, but this low C\textsubscript{60} concentration 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\textsubscript{60} was only less significant in primary astroces then in transformed glial cell, but it has no absent [38]. Distinct cytotoxic mechanisms were observed in respect with anticancer effect of pristine C\textsubscript{60} fullerene and water-soluble polyhydroxylated fullerene. Despite necrotic effect of pristine C\textsubscript{60} nanocrystals, hydroxylated fullerene C\textsubscript{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\textsubscript{60} nanoparticles exhibit extremely various bioactivity depending on the dose, the manners for solubilization and the rate of hydration of C\textsubscript{60} nanoparticles, which are critic for cytotoxic and cytoprotective features of C\textsubscript{60} fullerene and its derivates. Correlation between autophagy flux and paranhatos observed in our study uncovers the part of antiglioma effect of pristine hydrated C\textsubscript{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\textsubscript{60} on cell death machinery could be involved, at least partially, in the modulation of autophagy and paranhatos. Presented results demonstrate dose-dependent cytotoxicity of water-soluble HyC\textsubscript{60}Fn in U373GM cells. Furthermore, the doses of 1.0 and 2.0 \(\mu\text{M}\) HyC\textsubscript{60}Fn could initiate anti-tumor effect via the combination of severe autophagy flux and parthanatos in glioblastoma cells. Therefore, pristine hydrated C\textsubscript{60} fullerene displays potent anti-cancer features and further study is required.

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