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Supramolecular Insights into Domino Effects of Ag@ZnO-Induced Oxidative Stress in Melanoma Cancer Cells

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Abstract

Recent studies suggest that cancer cell death accompanied by organelle dysfunction might be a promising approach for cancer therapy. The Golgi apparatus has a key role in cell function and may initiate signaling pathways to mitigate stress and, if irreparable, start apoptosis. It has been shown that Golgi disassembly and fragmentation under oxidative stress act as indicator for stress-mediated cell death pathways through cell cycle arrest in the G2/M phase. The present study shows that UV-induced reactive oxygen species (ROS) generation by Ag@ZnO nanoparticles (NPs) transform the Golgi structures from compressed perinuclear ribbons into detached vesicle-like structures distributed in the entire cytoplasm of melanoma cells. This study also demonstrates that Ag@ZnO NPs-induced Golgi fragmentation co-occurs with G2 block of the cell-cycle progression, preventing cells from entering the mitosis phase. Additionally, the increased intracellular ROS production triggered by Ag@ZnO NPs upon UV exposure, promoted autophagy. Taken together, Ag@ZnO NPs induce stress-related Golgi fragmentation and autophagy, finally leading to melanoma cell apoptosis. Intracellular oxidative stress generated by Ag@ZnO NPs upon UV irradiation may thus represent a targeted approach to induce cancer cell death through organelle destruction in melanoma cells while fibroblast cells remained significantly unaffected.
Introduction

Despite extensive research efforts, current therapeutic approaches used for cancer have considerable drawbacks. Surgery, irradiation, and chemotherapy strategies have severe side effects \(^1-^3\). In light of these disadvantages, researchers are in search for new alternatives to eradicate cancer cells with maximal therapeutic and minimal adverse effects. It has been proposed that the inherent limitations of conventional therapies may be overcome by the use of nanoparticles (NPs) based drug delivery \(^4-^5\).

Apart from the conventional use of NPs as drug carriers, certain inorganic materials in nanoparticulate form, such as iron oxide, can directly induce oxidative stress, mitochondrial dysfunction, DNA damage, and other signalling pathways, leading to programmed cell death\(^6\). Also carbon based materials and silver nanoparticles have been shown to prevent cell proliferation, growth and migration, as well as angiogenesis in tumor region \(^7-^8\). Direct cytotoxic effects of nanoparticulate materials are mainly related to the release of toxic ions and/or their ability to induce oxidative stress \((i.e.,\) reactive oxygen species, ROS) \(^9-^10\). While a moderate level of ROS is critical for signal transduction and physiological process in cell physiology, an excessive amount of ROS damages biomolecules and causes cell death \(^11\). High levels of ROS induces cell cycle arrest and apoptosis in cancer cells. It also induces inflammation and interferes with autophagy \(^6,^12\). Oxidative stress is known to induce organelle malfunction. For example, DNA and Golgi fragmentation, mitochondrial dysfunction, and membrane disruption have been observed in cells subjected to oxidative stress \(^13-^14\). Golgi senses DNA damage mediated by oxidative stress, shows structural changes in response to stress, and triggers signalling pathways involved in the repair or cell death \(^15\). Like mitochondria, the Golgi apparatus can induce signals as a downstream consequence of programmed cell death mediated by oxidative stress \(^16-^17\). More importantly, the golgi apparatus has a regulatory role in cell division, so that cells with the fragmented golgi are not able to
undergo mitosis. Since cell division plays the most vital role in cancer cell survival, invasion and progression, golgi fragmentation following high amount of intracellular oxidative stress would arrest the cell cycle in cancer cells. So far, induced oxidative stress and corresponding organelles disruption and cell damage have been mainly studied for small molecules, while it has not been a primary field of cancer nanomedicine.

Due to their high metabolism, cancer cells are typically characterized by an intense amount of ROS, because of increased enzymatic activity of oxidases. Meanwhile, cancer cells neutralize adverse effects of excessive ROS via overexpressing the proteins involved in antioxidant defense system. However, exceptionally high levels of ROS may exceed the buffering capacity of the antioxidant defense system and lead to cancer cell death. Therefore, increasing the level of ROS or suppressing the antioxidant defense system in cancer cells could be an effective strategy to eradicate cancer cells. Recent studies have reported promising preliminary results on ROS boosting anticancer therapy. However, the concept of exploiting inducible ROS generation by NPs in order to induce cancer cell death, has not been investigated in detail yet. Recently, Therapeutics based on ROS generating NPs, capitalize on inducible ROS generation, has gained increasing attention. Previously, we have shown that core/shell NPs of Ag and ZnO (Ag@ZnO NPs) led to increased ROS generation, upon exposure to UV irradiation, representing a promising candidate for ROS-mediated cancer cell death.

Here, we report the application of Ag@ZnO NPs to induce organelle damage and cell cycle arrest in melanoma cells via increasing intracellular ROS level. The superficial anatomical location of melanoma cancer along with photocatalytic properties of Ag@ZnO NPs under UV irradiation, have the prospect to lead to an effective treatment approach (photodynamic therapy). We present how Ag@ZnO NPs can be employed for UV-inducible intracellular ROS generation, leading to autophagy and apoptotic cell death in melanoma cells.
Materials and Methods

Synthesis of Ag@ZnO Core-Shell Nanoparticles. Ag@ZnO NPs were synthesized using a wet chemical route as previously described. Briefly, Ag NPs were suspended in 0.06 M zinc acetate/DI solution and stirred for 30 min, then were added to NaOH/ methanol (0.4 M) and stirred at 90°C for 10 min. Following six washes, the obtained sample was calcined for 1.5 h at 550°C in a muffle furnace under atmospheric condition. Then, the samples were mortared to obtain fine powder.

Energy-Dispersive Analysis of X-rays (EDX). EDX spectrometer of Bruker XFlash-5030, liquid nitrogen-free silicon drift detector with optimized collection solid angle of ~0.13 sr was utilized in this experiment. The spectra were obtained with scanning electron microscope (SEM) equipped with Gatan MonoCL3 worked at 15 KV (JEOL JSM-6500F).

X-ray Photoelectron Spectroscopy (XPS). The spectra were collected using Escalad5, U.K. spectrometer, in an ion-pumped chamber (evacuated down to 2 × 10⁻⁹ torr). X-ray source of Al-Kα radiation with energy of 1486.6 eV was employed. Binding energy (BE) of spectra was calibrated by setting the measured BE of C 1s to 285 eV.

Cell Lines. Two types of human cell lines were used for in vitro studies: A375 (melanoma cancer, ATCC) and normal dermal fibroblasts. Cultured in DMEM media in 37°C with 95% O₂ and 5% CO₂.

UV Radiation. For UVB (H2 filter, 290-320 nm) radiation a lamp with power of 450 W was used. All experiments under UV radiation were done in a field-focus distance of 40 cm and 180 second radiation exposure.
Cell Treatment. NPs were added to the cell media after 24 h culture and cells were incubated for 2 h with 5, 10, 20, 30, 40, 50 and 100 µg/ml of Ag@ZnO NPs. NPs-treated and untreated cells were subsequently exposed to UVB radiation for 180 second under the mentioned lamp.

Fluorescent Labelling of the Nanoparticles and uptake study by Confocal Microscopy. In order to assess the cellular uptake of Ag@ZnO NPs, Rhodamine-B isothiocyanate (Rh-B) fluorescent dye (Merck, Germany) was conjugated to NPs following a previously published protocol. Then Rh-B-labelled-NPs (50 µg/ml) were dispersed in water and added to cells (n=1×10⁶). Uptake of NPs was assessed by flow cytometry, every 10 min during a total incubation time of 100 min, to identify an optimum uptake time scale. Uptake of Ag@ZnO NPs in both cell lines was also assessed by confocal laser scanning microscope (CLSM, Zeiss, Germany). Prior to imaging, cells were fixed and permeabilized with 4% formaldehyde and 0.2% Triton X 100 for 20 min at room temperature (RT). Cell nuclei were stained with DAPI (4", 6-diamidino-2"-phenylindole dihydrochloride; Sigma-Aldrich, USA). Samples were cultured on 35 mm circular glass bottom microwell dishes (MatTek, Ashland, USA). Alexa Fluor 488 Phalloidin was used to stain the actin skeleton.

Cytotoxicity Assessment.

Lactate Dehydrogenise (LDH) Assay. Toxic effect of Ag@ZnO NPs on melanoma cells and healthy fibroblasts has been assessed by LDH Assay (CytoTox 96® NonRadioactive Cytotoxicity Assay, Promega) following the manufacturer's protocol. Briefly, the cells (n=1×10⁵) were seeded in 96 well plates and incubated overnight. Then, different concentrations of Ag@ZnO NPs (10, 20, 30, 40, 50, 100 µg/ml) were added to cells and incubated for 2 h before exposure to UV radiation. Then, 50 µL of supernatant of each well was transferred to a new 96 well plate and incubated for 30 min in dark conditions with 50 µL
of CytoTox 96 reagent. Stop solution was added to each well and OD of each well was measured at 490 nm with a Mithras plate reader.

**Optical Microscopy.** Optical microscopy was used to visualize effects of NPs on cell morphology. In this regard, the cells (n=1×10⁶) were incubated for 24 h. Then, they were treated with different concentrations of Ag@ZnO NPs and incubated for an additional 2 h. Optical microscopy images have then been recorded (Zeiss, Germany).

**Annexin V/PI assay.** Cell apoptosis and necrosis induction by Ag@ZnO NPs was assessed using propidium iodide (PI) and Annexin V staining. Specifically, externalization of phosphatidylserine as a marker of early-stage apoptosis was detected by annexin V protein conjugated to FITC, whereas membrane damage due to late-stage apoptosis/necrosis was detected by binding of PI to nuclear DNA. After treatment with NPs either in dark condition or under UV radiation, cells (n=1×10⁶) were collected, washed with binding buffer (10 mM HEPES, pH 7.4; 140 mM NaCl; 2.5 mM CaCl₂) and incubated for 20 min on ice with 100 μl binding buffer containing Annexin V-FITC (40 μl/ml) and PI (1 μg/ml). Afterwards, 400 μl binding buffer was added to each sample, and cells were kept on ice. FITC (FL-1 chanel), and PI (FL-2 chanel) were evaluated by flow cytometry. For each sample, 10⁶ cells were analyzed and apoptotic (Annexin V+, PI−), necrotic (Annexin V+, PI+) and live (Annexin V−, PI−) cells were expressed as percentages of total cells. Cadmium sulfate and Staurosporine were used as positive controls for necrosis and apoptosis, respectively.

**Oxidative stress assessment**

**In-solution ROS.** DPPH (1,1-Diphenyl-2-picrylhydrazyl), as a total radical scavenger was used to measure in-solution ROS ²⁵. The DPPH radical scavenging activity was calculated from equation 1 in which H and H₀ indicate optical density of the solvent with and without sample, respectively.
Radical scavenging activity (%) = \[\frac{(H - H_0)}{H_0} \times 100\]  \hspace{1em} (Equation 1)

**Type of intracellular ROS.** Intracellular ROS generation by Ag@ZnO on melanoma cells, was evaluated by flow cytometry and confocal microscopy using H$_2$DCF-DA (2′,7′-dichlorodihydrofluorescein diacetate, Sigma, USA) and DHE (Sigma, USA) specific probes for H$_2$O$_2$ and O$_2^-$, respectively, in dark conditions and under UV radiation. H$_2$DCF (25 mM) and DHE (1.25 mM, Sigma-Aldrich, USA) were added to the cells for 1 h, then, cells were washed twice with HBSS before treatment with Ag@ZnO NPs. Hydrogen peroxide (H$_2$O$_2$) was used as positive control. Green (DCF) and red (HE) fluorescence were assessed in the range of 500-530 nm, and 590-700 nm, respectively (excitation, 488 nm; emission, 525–625 nm in the FL-2 channel). The populations of cells were gated using 90° and forward angle light scatter to distinguish debris and aggregates. DCF green fluorescence and HE/PI emitting red fluorescence (580–630 nm) were recorded in the FL-1 and FL-2 channels, respectively.

**Confocal Microscopy for intracellular ROS.** Melanoma cells \((n=1 \times 10^6)\) were treated with 50 µg/ml of RhB-labelled Ag@ZnO NPs under UV radiation, and ROS generation was assessed by Confocal microscopy. H$_2$DCF (25 mM) was added to cells and incubated for 1 h, then the cells were treated with Ag@ZnO NPs and exposed to UV radiation. The cells were washed with PBS, fixed with 4% formaldehyde (4% PFA) and imaged.

**Glutathione Peroxidase (GPx) Assay.** Activity of glutathione peroxidase in healthy and cancerous cells after exposure to Ag@ZnO NPs under UV radiation was evaluated with a commercially available Glutathione Reductase Assay Kit (GRSA, Sigma Aldrich, USA), in accordance with the manufacturer’s instructions.

**Superoxide Dismutase (SOD) Assay.** SOD Assay Kit (Abcam, USA) was utilized to assess the superoxide dismutase enzyme level in cells after treatment with Ag@ZnO NPs, according to manufacturer’s protocol.
**Catalase (CAT) Assay.** Catalase Activity Assay Kit (Abcam, USA) was utilized to assess the catalase enzyme level in cells after treatment with Ag@ZnO NPs, according to manufacturer’s protocol.

**Study of Golgi structure by confocal microscopy.** The cells were labelled with anti-GM130 antibody (CellLight Golgi-GFP, Thermo Fisher) to monitor organization of Golgi membranes, and with DAPI for nuclei staining based on the manufacturer’s protocol. Shortly, the cells were plated at 1×10^6 cell/well density. After 24 h of culture, anti-GM130 antibody was added directly to cell media. After an overnight incubation (>16 h), cells were treated with 50 µg/ml of Ag@ZnO NPs for 2h and they exposed to UV for 180 seconds. Then, the samples were fixed with 4% PFA in PBS, followed permeabilization with 0.2% Triton X100 for 5 min.

**Quantification of Golgi fragments.** Image J software was utilized for quantitative analysis of Golgi fragments in confocal micrographs. The fluorescence intensity of fragmented Golgi objects (based on GM130 staining) was determined by background subtraction and using a fixed threshold in a mean 100 cells. Then the fragmented particles which dispersed in cytoplasm were quantified using the Analyze Particle plugin in Image J. 27

**Evaluation of GRASP65 Protein Level.** GRASP65 Protein level in melanoma cells after treatment with Ag@ZnO NPs and UV radiation was evaluated using a Human GRASP65/GORASP1 ELISA Kit (Fine Test, USA), according to manufactures protocol.

**Autophagy Assessment.** Autophagy in Ag@ZnO NPs-treated melanoma cells under UV radiation, has been assessed using an autophagy assay kit (Abnova). Briefly, the 10^5 cells/well were cultured 24 h in 35 mm glass button flask and treated with NPs for 2 h, then medium has been removed and 1 mL of Autophagy blue working solution has been added to the cells for a 30 min incubation. Then, cells were washed and fixed with 4% PFA. The nucleus of cells was stained with DRAQ5 red fluorescent and evaluated by confocal Microscopy (Zeiss, Germany).
To evaluate the concentration-dependent effect by NPs on Autophagy, the cells were washed with PBS and analyzed at Ex/Em= 335/520 by ELISA reader.

**Cell Cycle Analysis by PI flow cytometry.** Cells (n=1×10^6) were exposed to 0, 20, 30, 40, 50 and 100 µg/ml of Ag@ZnO NPs for 2 h and, after UV radiation exposure, stained with PI, for the cell cycle analysis. For this experiment, cells were harvested and fixed in 70% cold ethanol for 30 min at 4°C. After washing with PBS, the cells were treated with 50 µl of RNase to ensure only DNA staining. Then, 200 µl of PI were added to the samples and the fluorescence intensity was measured with a flow cytometry. Forward and side scatters were measured to identify the single cell with an emission wavelength of 610 nm. Data collected for 1x10^6 cells was analyzed using Multi Cycle AV for Windows software (Phoenix Flow Systems, San Diego CA. USA).

**Results and Discussion**

Synthesis of Ag@ZnO nanoparticles was carried out according to our previous report 28. Transmission electron microscopy (TEM) and small angle X-ray scattering (SAXS) of Ag@ZnO nanoparticle showed an average particle size of 16 nm for Ag core and mean thickness of 3 nm for ZnO shell with spherical morphologies. Moreover, XRD pattern confirmed hexagonal wurtzite structure of crystalline ZnO shell, where UV-Vis spectar also confirmed formation of core-shell structure 25. EDS analysis showed that atomic ratio of Zn/Ag is 2.4 (Figure S1). Furthermore, X-ray photoelectron spectroscopy was carried out to confirm the presence of Ag and Zn elements and purity of Ag@ZnO NPs (Figure S2).

**Ag@ZnO Uptake in Melanoma and Fibroblast Cells**

Cytotoxic effects of NPs often occur following their internalization into cells. First, we evaluated the cellular uptake of Rhodamin B (Rh-B)-labelled Ag@ZnO NPs in normal human
dermal fibroblasts and melanoma cells. The conjugation of Ag@ZnO NPs with Rhodamin B was confirmed by UV-vis spectroscopy. Then, uptake of the Rhodamin B-labelled Ag@ZnO NPs into dermal fibroblasts and melanoma cells was monitored for 100 minutes of incubation, and the cellular uptake was semi-quantitatively measured by flow cytometry (Figure 1A). The fluorescence results indicate that the uptake of Ag@ZnO in melanoma cells reached a plateau after 70 min incubation where 96% of the cell population showed significant increase in mean fluorescence (Figure 1C). At the same time, 68% of the fibroblasts showed a change in fluorescence intensity, also reaching a plateau (Figure 1B). Most of uptake mechanisms are non-specific, so the healthy cells uptake NPs during incubation, even though cancer cells uptake more NPs due to their higher metabolism\textsuperscript{29}. The uptake of Ag@ZnO NPs was also assessed after 70 minutes of incubation by fluorescence imaging using laser confocal scanning microscopy (CLSM). The confocal images suggest slightly higher uptake of Ag@ZnO into that melanoma cells compared to the fibroblasts (Figure 1D). Confocal fluorescence images indicate that Ag@ZnO NP are predominantly found in the perinuclear region in the melanoma cells. The size of NPs represents one of the determining factors for cellular uptake\textsuperscript{30}. Previous reports state that 50-80 nm NPs are mostly taken up by endocytosis route\textsuperscript{31}. The Ag@ZnO NPs present a size of around 70 nm in cell culture media and can therefore be readily taken up by endocytosis pathways\textsuperscript{25}. While uptake is observed in both healthy and cancerous cells, increased internalization is observed in cancer cells due to their higher metabolism\textsuperscript{29}. After confirming the uptake of the Ag@ZnO NPs into the melanoma cells, secondary effects, including potential damage to organelles need to be assessed in detail. It is required to study the potential impacts of Ag@ZnO at molecular and organelle level, in order to understand the mechanisms by which Ag@ZnO-induce oxidative stress.
Figure 1. Time-dependent uptake of 50 ppm Ag@ZnO in healthy dermal fibroblast and melanoma cancer cells, evaluated by flow cytometry every 10 minute during 100-minute incubation (A). Histogram plot of Ag@ZnO in dermal fibroblast (B) and melanoma (C) cells after 70 minutes of incubation as an optimized time point for preferential uptake into melanoma cells. Laser confocal micrograph images for qualitative assessment of NPs uptake in melanoma and dermal fibroblast cells using Rh-B conjugated Ag@ZnO NPs. The cytoskeleton is stained by Alexa Phalloidin A488 (green); nuclei are stained by DAPI (blue) (D).

UV Irradiation Amplifies the Cytotoxicity of Ag@ZnO in Melanoma Cancer Cells

To investigate whether the oxidative stress elicited by Ag@ZnO NPs upon UV exposure leads to cytotoxicity in cells, viability of healthy and cancer cells treated with Ag@ZnO NP were
assessed under UV irradiation and in dark conditions (without UV exposure). Lactate dehydrogenase (LDH) assays were used to measure cell death using LDH as a surrogate marker for membrane disruption in dermal fibroblasts and melanoma cancer cells (Figure 2A and B). Cell death in dark condition results negligible for both fibroblasts and melanoma cells. On the contrary, upon UV irradiation, Ag@ZnO NPs exert a concentration-dependent toxic effect of on dermal fibroblasts, reaching about 50% cell death at the higher concentration used (100 µg/ml) (Figure 2A). Melanoma cells were even more sensitive, showing a 50% cell death when incubated with 30 µg/ml of Ag@ZnO NPs, and 100% cell death with the maximum dose (100 µg/ml) used. Thus, melanoma cancer cells are much more sensitive than dermal fibroblast, to high amount of oxidative stress due to their higher uptake, higher metabolism, and lower antioxidant capacity. Moreover, the morphology of the cells treated with Ag@ZnO NPs changed from elongated to rounded structures following UV irradiation (Figure 2C and S3). Indeed, while melanoma cells treated with Ag@ZnO NPs in a dark condition, showed only minor cytotoxicity (≤10% for concentrations of 50 µg/mL or lower (Figure 2B), the UV-driven effect is strongly amplified, in a dose dependent manner, in melanoma cells incubated with Ag@ZnO NPs. Previous reports on photodynamic therapy against colon cancer with Chlorin e6 photosensitizer and melanoma cancer with phthalocyanine-conjugated gold NPs, showed a gradually/concentration dependent rounding up of cells, in presence of light, similar to the effects observed in the present study. Literature showed that the cells lose their characteristic morphology, round up, detach and start to be free-floating in media after photodynamic therapy. These observations are here confirmed by flow cytometry analysis, also indicating that both the number of apoptotic and necrotic cells are considerably higher in cells treated with nanoparticles under UV irradiation compared to dark conditions (Figure 2D-F and S4). Also, dermal fibroblast cells have undergone apoptosis and necrosis, but to a lower extent (Figure S7). These results indicate that toxic effects of Ag@ZnO NPs on melanoma cells
are primarily related to their potency to produce ROS under UV irradiation. The UV-induced ROS generation leads to oxidative stress that activates signalling pathways triggering apoptosis. By this method, we are able to target the lesion area on the skin by focusing the irradiation. So, it is possible to activate the NPs which are uptaken by cancer cells and do not excite those who are uptaken by the healthy cells (out of the focusing area). In this regard, the high amount of toxicity of NPs in cancer cells upon UV exposure should be compared to the moderately toxic effect of NPs in unexposed area containing healthy cells.

**Figure 2.** Cytotoxicity evaluation of different concentrations of Ag@ZnO NPs, toward dermal fibroblasts (A) and melanoma cancer cells (B), in dark conditions and under UV irradiation, using LDH assay. Optical micrograph of melanoma cells after treatment with 10, 30, 50 and 100 µg/ml of synthesized NPs in the presence of UV irradiation, along with untreated and UV-treated cells as controls (C). Percentage of cell death (necrosis and apoptosis) by different concentration of Ag@ZnO NPs, in dark conditions (D) and under UV irradiation (E). Representative scatter plot illustration of flow cytometry studies of cell death using annexin V-PI kit (F).
Ag@ZnO NPs Stimulate ROS Production

Following the hypothesis that the anticancer properties of Ag@ZnO NPs are likely related to their ability to induce oxidative stress, in the first step, we studied the cellular oxidative stress induced by Ag@ZnO in healthy dermal fibroblasts and melanoma cells. Different concentrations of Ag@ZnO were incubated with melanoma and skin fibroblast cells under UV to investigate NP-induced oxidative stress via measuring levels of ROS (H$_2$O$_2$ and O$^\cdot$$_2$) (Fig. 3A-C). Additionally, the activity of enzymes involved in antioxidant defence system were measured (e. g., superoxide dismutase (SOD), glutathione peroxidase (GPX) and catalase (CAT), Fig. 3D, E, and F).

Depending on type, level, and locations in the cells, elevated ROS leads to different oxidative stress-mediated damages. Therefore, it is required to determine the ROS types and level produced in the presence of NPs. H$_2$ DCFDA and HE are the most common probes used for detection and quantification of H$_2$O$_2$ and O$^\cdot$$_2$, respectively. The fluorescent microscopy images confirmed the generation of ROS in Ag@ZnO-treated cells (Figure 3A). As observed in Figure 3B, low concentrations of Ag@ZnO NPs increased H$_2$O$_2$ level in melanoma and fibroblast cells, upon UV exposure. For concentration of 30, 40 and 50 µg/ml, a higher amount of H$_2$O$_2$ was produced in melanoma cells compared to fibroblasts due to higher antioxidant capacity of healthy cells to quench excessive radicals. The HE test showed that, at high concentrations (i. e. 50 and 100 µg/ml), Ag@ZnO NPs could increase O$^\cdot$$_2$ level in the cells (Figure 3C). As a conclusion, Ag@ZnO induces oxidative stress via generating H$_2$O$_2$ and O$^\cdot$$_2$ under UV irradiation. The results indicate that Ag@ZnO NPs induce substantial oxidative stress in melanoma cells (results for control groups in PBS buffer and under in dark condition and under UV irradiation are detailed in Figure S5 and S7). Also ROS generation by as prepared ZnO NPs in melanoma cells in dark condition and under UV radiation is reported as Figure S6 in
SI file. ROS generation of the synthesized NPs could result in mutations and oxidative stress-related cell damages.\textsuperscript{39-40} Oxidative stress could trigger DNA damage and cause subsequent cell death in cancer cells.\textsuperscript{41-42} Previous research using iron oxide NPs indicated that excessive amount of ROS production is related to autophagy in cancer cells.\textsuperscript{6,43}

SOD, CAT, and GPX are the first-line antioxidants that neutralize ROS and prevent the production of free radicals. SOD converts superoxide radicals into $\text{H}_2\text{O}_2$ and either molecular oxygen or $\text{H}_2\text{O}_2$. Catalase (CAT) decomposes hydrogen peroxide to water and oxygen and Glutathione peroxidase reduces free hydrogen peroxide to water.\textsuperscript{44} Here, the activity of these enzymes were assessed in melanoma cells following Ag@ZnO NPs treatment and UV irradiation. SOD, GPX, and CAT significantly increased when cells were incubated with increasing concentrations of Ag@ZnO NPs, specifically from 30 to 50 $\mu$g/ml. The highest antioxidant activity was observed at a NPs concentration of 50 $\mu$g/ml (Figure 3 D-F). Melanoma cells died after incubation with a NPs concentration of 100 $\mu$g/ml, and hence, no enzymatic activity is observed. Results for control cells in dark condition are shown in Figure S8. The role of metal NPs triggered oxidative stress in disruption of cell haemostasis and consequent antioxidant activity were investigated in previous research.\textsuperscript{45-46} Accordingly, the excessive amount of ROS triggered by Ag@ZnO NPs in cells under UV irradiation could disrupt the antioxidant capacity of cancer cells and direct them to cell death mechanisms.
Figure 3. Intracellular ROS generation of Ag@ZnO NPs (50ppm)-treated melanoma cells, detected by DCFDA probe and captured with laser confocal microscopy in comparison with untreated, and H$_2$O$_2$-treated cells, as negative and positive control, respectively. DRAQ5 (red) is used for nucleus staining (A). Detection of DCF (B) and HE (C) to quantitatively determine generated H$_2$O$_2$ and O$_2$ radicals, in healthy dermal fibroblasts and melanoma cancer cells, after treatment with different concentrations of NPs, Statistics are compared with un-treated control. Determination of oxidative stress enzymes after treatment of melanoma cells with different concentrations of NPs by superoxide dismutase (D), glutathione peroxidase (E) and catalase (F) assays.

Oxidative Stress Induced by Ag@ZnO Leads to Golgi Fragmentation and Autophagy

The Golgi apparatus is one of the most essential cellular organelles that modifies, sorts and packages proteins and lipids in the cells as well as orchestrates intracellular distribution and secretion of biomolecules. Many of signalling pathways are regulated by Golgi apparatus, which also plays a crucial role in controlling the entry of mammalian cells into mitosis. In this regards, phosphorylation of GRASP65 protein is essential for mitotic Golgi fragmentation, which converts perpendicular Golgi stacks into fragments during mitosis. Therefore, Golgi can be an attractive target to interfere with the cell vital processes and mitosis. It is well
understood that Golgi apparatus alters in response to oxidative stress-induced cell damage\textsuperscript{15}. Golgi morphological changes associated with signalling pathways trigger cell repair or apoptosis. The proteins GRASP65 and GM130 are main structural components of the Golgi membrane that play a crucial roles in Golgi stacking and integrity\textsuperscript{50-51}. These proteins are widely targeted to evaluate Golgi fragmentation. To investigate whether Ag@ZnO-induced oxidative stress affects Golgi integrity, we probed the GM130 pattern and GRASP65 expression using immune-based assays. GM130-GFP probe was used to evaluate the Golgi integrity in cells. As shown in Figure 4A, melanoma cells treated with Ag@ZnO NPs have fragmented Golgi distributed around nucleus, while control cells have compressed perinuclear ribbon-like Golgi (Figure S9). Moreover, the number of cells with fragmented Golgi (Figure 4B) and amount of Golgi fragments/cell (Figure 4C) are increased in a concentration dependent manner, upon UV exposure. Literature reveals that breakdown of the Golgi apparatus is a significant consequence of oxidative stress damage to the cells and is known as one of the early events during apoptosis\textsuperscript{52}. Golgi reversibly fragments during apoptosis induced by oxidative stress which are similar to mitosis, even though the involved mechanisms are fairly different\textsuperscript{19}.

GRASP65 links to the Golgi structure and changes due to integration signals, in order to undergo mitosis or start apoptosis\textsuperscript{53}. The expression of GRASP65 protein is significantly reduced in cells exposed to Ag@ZnO (Figure 4D). Under-expression of GRASP65 enhances the kinetics of Golgi fragmentation due to oxidative stress and hence, Golgi structure senses and regulates initial events in apoptotic cell death\textsuperscript{52}. These findings indicate that Ag@ZnO-induced oxidative stress triggers Golgi fragmentation in melanoma cells. The Golgi disassembly oftentimes co-occurs with other alterations of biological processes (e. g. autophagy and cell cycle arrest) and may induce cancer cell apoptosis (Figure 4E).
Autophagy is a self-degradative process that delivers cytoplasmic components or fragmented organelles to the lysosome in order to prevent apoptosis and restrict cell necrosis. This intracellular degradation system plays a vital role in recycling misfolded proteins, damaged organelles, and pathogens in response to oxidative stress. It is reasonable to hypothesize that fragmented Golgi and damaged biomolecules, due to oxidative stress, can stimulate autophagy processes. To check this hypothesis, the autophagy process in cells treated with Ag@ZnO NPs were assessed, in comparison with Rapamycin-treated cells, as positive control. As shown in Figure 4F and G, fluorescent blue autophagy dye significantly increased in cells treated with increasing concentration of Ag@ZnO NPs. Also, UV-treated cells and Ag@ZnO NPs-treated cells in dark condition have not shown any specific fluorescence of autophagy, in comparison with Rapamycin, which led to a 100% autophagy induction (Figure S10). Studies showed that the loss of Golgi ribbons in cells results in autophagosomes formation in order to prevent apoptotic/necrotic cell death. Therefore, it can be suggested that the fragmented/damaged organelles resulting from oxidative stress activate autophagy in cells after treatment with Ag@ZnO NPs. However, cells with fragmented Golgi may survive through autophagy. Cell death due to extreme oxidative stress should happen as the consequence of unsuccessful adaptation.
Figure 4. Confocal micrograph of the Golgi structure before and after treatment of melanoma cells with Ag@ZnO NPs using GM130-GFP and DAPI (A). Evaluation of 100 cells with fragmented Golgi (B) and fragments/cells (C). Measurement of GRASP65 protein in melanoma cells after treatment with 20 and 50 ppm of Ag@ZnO NPs (D). Schematic representation of the assessed pathway which results in Golgi fragmentation and consequent autophagy (E). Quantitative assessment of Ag@ZnO NPs induced autophagy in melanoma and dermal fibroblast in a concentration-dependent manner, statistics are in comparison with untreated control (F). Qualitative visualization of autophagosomes formation in melanoma cells after treatment with NPs, DRAQ5 (red) is used for nucleus staining (G).
Oxidative Stress Induced by Ag@ZnO leads to Cell Cycle Arrest

Cancer cells are associated with genetic mutations, leading to the dysfunction of regulatory processes and uncontrolled cell proliferation. After years of research on cell cycle proteins and on their significance in cancer progression, cell cycle arrest became one of the most effective strategies used for cancer therapy. Moreover, studies showed that the inhibition of Golgi disassembly and related pathways, results in cell cycle arrest at the G2 stage. In order to assess cell cycle arrest caused by Ag@ZnO NPs, flow cytometry analysis was performed in cells exposed to Ag@ZnO NPs. (Fig. 5A and B). This analysis shows that Ag@ZnO NPs arrest the melanoma cycle at G2/M phase in a concentration dependent manner, with the best result obtained after treatment with 50 µg/ml of NPs. Other studies showed that ZnO, nickel, TiO$_2$ and silver NPs have the potential to arrest cancer cells in the G2/M phase. Our results confirm the potential effects of Ag@ZnO NPs to induce cell cycle arrest in melanoma cancer cells upon UV exposure. The cell-cycle arrested cells could not enter into the mitosis phase, hence Ag@ZnO NPs could be considered as a novel therapeutics for stopping cancer cell division.

Furthermore, cyclin and cyclin-dependent kinases (CDKs) are the most important regulators of the cell cycle processes. CDKs bind to a cyclin, and the resulted complex trigger cell cycle. CDKs, as engine of the cell cycle, are attractive targets for cell cycle arrest. CDK1 is the primary regulator that joins to cyclin A and cyclin B to drive cells through G2 phase and mitosis, respectively. Moreover, CDK1 is a key mitotic kinase and it has been shown that Cdk1–cyclin B is the major kinase phosphorylating GRASP65 in mitosis. Researchers have suggested a model in which the CDK1 phosphorylation event creates a landing platform for GRASP65 that in turn phosphorylates the serine residue situated in the GRASP second PDZ domain, thus explaining the link between C-terminus phosphorylation events and Golgi ribbon unlinking at mitosis. Using an ELISA assay, we showed that the expression of CDK1 in cells
treated with Ag@ZnO NPs is significantly reduced (Fig. 5C), confirming that Ag@ZnO can arrest cancer cell cycle in G2/M phase. Figure 5D schematically represents the processes involved in cell cycle arrest, which starts by a high amount of ROS and continues with Golgi fragmentation and CDK1 reduction to end in G2/M cell cycle arrest where the Golgi is unable to receive the mitosis signal. The findings showed that the direct inhibition of CDK1 may cause damage to normal cells along with cancer cells, while mechanistic regulation of CDK1 with NPs result in direct G2/M phase cell cycle arrest.

Figure 5. Flow cytometry study of the cell cycle after treatment of melanoma cells with different concentration of Ag@ZnO NPs (histograms) (A), quantitative percentage of cells in each cell cycle phase after treatment with NPs (B). Evaluation of amount of CDK1 protein level in cells after treatment with 20 and 50 ppm of NPs in comparison with non-treated melanoma cells (C). Schematic representation of the effect of ROS generation on melanoma cells and the chain reaction of the cells which results in cell cycle arrest in G2/M (D). All samples are compared with untreated control and * is referred to $P \leq 0.05$, ** for $P \leq 0.01$ and *** for $P \leq 0.001$. 
Conclusion
The present research showed that generation of ROS by Ag@ZnO NPs under UV irradiation can disrupt melanoma homeostasis while this effect was significantly lower in fibroblast cells. Treatment with Ag@ZnO NPs goes along with Golgi fragmentation directing cells to undergo autophagy in order to survive, or initiate apoptosis. Golgi fragmentation triggered by excessive ROS could result in G2/M block of cell-cycle progression and prevent cancer cells from entering into the mitosis phase. If the cells can not compensate the excessive ROS generated by Ag@ZnO NPs by antioxidant adaptation, the cancer cells will undergo G2/M cell cycle arrest and apoptosis following the treatment with Ag@ZnO NPs and UV. In conclusion, Ag@ZnO NPs may hence be a promising topical therapeutic agent in the process of photodynamic therapy of melanoma, used to trigger intracellular ROS upon UV exposure and may be used to treat melanoma cancer.

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Supporting Information
EDS mapping of Ag@ZnO NPs, XPS spectra of Ag@ZnO NPs, optical microscopy of Ag@ZnO treated cells in dark condition, flowcytometry results for necrosis/apoptosis state of dermal fibroblast cells after treatment with Ag@ZnO NPs in different concentration, Control study for ROS generation under UV irradiation, and in Ag@ZnO NPs treated melanoma cells, DCF and HE fluorescence data for Ag@ZnO NPs treated cells in dark condition, results of oxidative stress related enzymes in dark condition, Golgi structural study for UV control samples and cells treated with Ag@ZnO NPs in dark condition, UV control for autophagy and
Ag@ZnO NP treated cells in dark condition along with Rapamycin treated cells as positive control.

**Author contributions**

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