Research Article

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Silver nanoparticles induce mitochondria-dependent apoptosis and late non-canonical autophagy in HT-29 colon cancer cells

https://doi.org/10.1515/ntrev-2022-0114
received January 2, 2022; accepted April 25, 2022

Abstract: The interactions of nanomaterials with biological materials such as immortalized cell lines are recently on the rise. Owing to this superiority, the biosynthesis of AgNPs using gallic acid as a reductant was implemented in this study. After being synthesized, the AgNPs were characterized using techniques such as dynamic light scattering, transmission electron microscopy, selected area electron diffraction, and X-ray diffraction methods. Furthermore, the AgNPs were assessed for their cytotoxic effects on the colorectal adenocarcinoma cell line HT-29. The mechanisms of such cell-killing effect were investigated by analyzing the expressions of 14 mRNAs using quantitative polymerase chain reaction. The outcomes indicate that the synthesized AgNPs were cytotoxic on HT-29 cells. The expressions of all apoptotic genes analyzed including cyt-C, p53, Bax, Bcl2, CASP3, CASP8, CASP9, and CASP12 were upregulated. With regard to the autophagy-related genes, Beclin-1, XBP-1, CHOP, and LC3-II were upregulated, whereas the expressions of ATG3 and ATG12 were downregulated. To conclude, the AgNPs induced mitochondria-dependent apoptosis and non-canonical autophagy in HT-29 cells. A crosstalk did occur between autophagy and apoptosis in such a cell-killing effect. Hence, further studies are required to elucidate the exact mechanisms in animal models for further use of AgNPs in clinical medicine for the treatment of neoplasms of the digestive tract.

Keywords: AgNPs, HT-29, cytotoxicity

1 Introduction

Nanotechnology and its roles in cancer therapy are intensifying due to their specific targeting properties, and this arena of research has the potential to overcome the limitations of conventional treatment methods [1]. The drawbacks of using chemical and physical means comprise the harmful methods that pose higher energy requirements and utilize synthetic reductants that can generate substantial amounts of waste products [2]. Biosynthesized AgNPs are better used in nanomedicine because of their properties including small size and low toxicity with increased biodegradability and availability [3–5]. Green synthesis is an established route for fabricating AgNPs anticipated to be used for applications in medicine [6,7]. It has several advantages over the nanoparticles synthesized using conventional chemical methods [8–12]. Particularly, AgNPs synthesized using biological methods are known to be effective against microbes such as bacteria, fungi, and viruses with wound-healing, anti-inflammatory, and antioxidant properties posing its candidacy for treating diseases like cancer and diabetes. In addition, the active capping agents of such nanoparticles fabricated using green methods are credited for the enhanced biological activities of such materials [13,14]. Colon cancer is one of the top five reasons for deaths related to cancers worldwide. In China, the incidence and
death percentage related to colorectal cancers have increased considerably in 2020 compared to that in the year 2015 [15]. In the United States, an estimated 338,090 new cases and 169,280 deaths are expected because of the cancers associated with the digestive system in 2021. In the same report, 149,500 new cases and 52,980 deaths are estimated to happen because of cancers of the colon and rectum [16]. According to the 2022 projections, an estimated 343,040 new cases and 171,920 deaths related to digestive cancers are likely to occur. Consequently, 151,030 new cases and 52,580 deaths related to cancers of the colon and rectum are projected for the year [17]. In vitro studies on cancer cell lines are cherished, as these models are noteworthy in studying the mechanism of cytotoxicity induced by a variety of genes related to oncogenic or oncosuppressive elements [18]. Thus, from the time of its identification in 1964, the human adenocarcinoma cell line HT-29 has been used as a molecular model for studies related to intestinal cancer [19].

The benefits of using cell lines as an alternative for animal models are that they are efficient in managing the cost of conducting an experiment, provide ease in conducting and applying the outcomes, offer a limitless source of material, and evade the ethical issues connected with the usage of animal and human tissues [20]. Assays that can determine the cytotoxic effects of a drug screening for its efficacy in inhibiting cellular proliferation are used in combination with molecular techniques such as real-time polymerase chain reaction (PCR). Studies of such kind aim at quantifying the expressions of genes related to oncogenic or oncosuppressive effects to study the mechanism of cytotoxicity induced by a drug [21].

Among mechanisms being studied, apoptosis is a widely accepted signaling pathway for the death of malignant cells and is an outcome of triggers initiated by internal or external stimuli [22]. Autophagy is another process by which cellular homeostasis is maintained. This mechanism results in forming a double-membraned autophagosome to engulf the unwanted cargo of organelles by the development of an autolysosome [23]. Although it is considered a double-edged sword, autophagy is predominantly a tumor suppressor in the early stages of a tumor [24]. It is a less-studied mechanism for analyzing the route of cytotoxic effects of AgNPs on cancer cells. Apoptosis and autophagy can cross-talk involving quite a lot of biological macromolecules. The interaction between these two mechanisms of cellular degradation and homeostasis determines the fate of a cell. These signals can therefore protect a host against several diseases including cancer [25].

Based on their cytotoxic effects at various levels via the displacement of Ag⁺ ions or the AgNPs as a whole, they are a part of several formulations (almost one-third) used in numerous industries. Properties such as size, dose, route of administration, and the capping agents play crucial roles in the absorption of AgNPs. The mechanisms for such effects are linked to damage of the hereditary materials, change in the intake of nutrients and the active roles of cells of the immune system, the stimulation of intracellular ROS-associated membrane damage, angiogenic effects, cell-cycle arrest, and the induction of apoptosis [26–30].

Based on this background, the AgNPs were synthesized using gallic acid, and the mechanisms of their cytotoxic effects against HT-29 colon cancer cells were determined. According to the published reports, this is the first-ever international study on the analysis of the cross-talk between autophagy and apoptosis as cell-killing mechanisms of AgNPs in colon cancer cells.

2 Experimental section

2.1 Chemicals

All chemicals used for this study were of analytical grade. Silver nitrate and gallic acid were purchased from Fisher scientific and Merck, USA, respectively. Dulbecco’s modified Eagle medium (DMEM), fetal bovine serum (FBS), and antibiotic solutions were obtained from Gibco (Grand Island, NY, USA). Dimethyl sulfoxide and 3,4,5-dimethylthiazol-2-yl-2,5-diphenyl tetrazolium bromide (MTT) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2 Synthesis and characterization of AgNPs

To synthesize AgNPs using gallic acid, different ratios of 0.01 N AgNO₃ and 0.1 M gallic acid solutions were mixed (5:5, 6:4, 7:3, 6:4, 8:2, and 9:1). The mixture was incubated at room temperature. The change in intensity of the color of the hydrosol toward brown was monitored over a period of 12 h and pictured at the end of the incubation. The solutions prepared by proportions of 5:5, 8:2, and 9:1 were analyzed using dynamic light scattering (DLS). Micromeritics model Nano Plus was used to determine whether the nanoparticles were synthesized at the highest (5:5) and the limited concentrations of gallic acid (9:1). The best ratio with respect to particle size was obtained at the least concentration of gallic acid used (9:1) and therefore was considered for the bulk synthesis of AgNPs intended in pursuit of further use. Transmission electron microscope
(TEM) images and selected area electron diffraction (SAED) patterns were obtained using JEOL JEM-2100F FE-TEM. Investigation of the crystalline nature of the material via X-ray powder diffraction (XRD) was accomplished using the PANalytical X’Pert® Powder instrument (Malvern Pananalytical Inc., Westborough, MA, USA).

2.3 Cell culture and MTT assay

HT-29 cells (human colon adenocarcinoma cell line) were cultured in DMEM with 10% FBS and penicillin/streptomycin (100 μg/mL) in a humidified incubator with a 5% CO₂ atmosphere at 37°C.

The cytotoxicity of AgNPs on HT-29 cells was tested using MTT assay. Briefly, the cultured HT-29 cells were harvested by trypsinization and pooled into a 15 mL tube. The cells were then plated at a density of 1 × 10⁴ cells/well (200 μL) into 96-well tissue culture plates with DMEM for 24–48 h at 37°C. Later, the wells were washed with sterile PBS and treated with various concentrations of AgNPs in a serum-free DMEM. The experiment was repeated three times, and the cells were incubated at 37°C in a humidified 5% CO₂ incubator for 24 h. After the incubation period, MTT was added to each well, and the cells were incubated for another 2–4 h and observed under an inverted microscope to evaluate the viability of HT-29 cells. The absorbance for each well was measured at 570 nm using a microplate reader (Thermo Fisher Scientific, USA). The percentage cell viability and IC₅₀ were calculated using GraphPad Prism 6.0 software (USA).

2.4 Optimization of cell viability by response surface methodology (RSM)

RSM was used to correlate the factors and optimum responses for cell viability. The central composite design (CCD) was used for optimization. Design-Expert® software (Version 12; State-Ease Inc., Minneapolis, MN, USA) was applied to infer the outcomes.

2.5 Real-time PCR to analyze the expression of mRNAs

After the IC₅₀ value was determined, the HT-29 cells were treated with the determined dose and real-time PCR was used to quantify the expression of 14 mRNAs preferred for the study. The cells were centrifuged at 5,000 rpm for 10 min in tubes treated with diethyl pyrocarbonate. The pelleted cells (1 × 10⁷ cells) were treated with TRIZOL (Sigma-Aldrich, St. Louis, MO, USA) to lyse the cells. After a purity check for DNA contamination has been done, cDNA was synthesized and real-time PCR was performed using SYBR® Green JumpStart™ Taq Ready Mix™ (Catalog Number S4438). The expressions of apoptotic and autophagy-related mRNAs such as BCL2 Associated X; Apoptosis Regulator (Bax); Bcl2 Apoptosis Regulator (Bcl2); cytochrome C (cyt-C); Tumor protein P53 (p53); caspases 3, 8, 9, 12 (CASP3, CASP8, CASP9, and CASP12); X-Box Binding Protein 1 (XBP-1); C/EBP homologous protein (CHOP); LC3-II; Beclin-1; Autophagy-related 3 (ATG3); and Autophagy-related 12 (ATG12) were analyzed. β-Actin was used for normalization of the expression of the gene of interest. The primers used for the analysis are enlisted in Table 1. After the primers and other necessary materials were obtained, a real-time PCR assay was conducted using StepOnePlus Real-Time PCR, Applied Biosystems.

2.6 Statistical analysis

The expressions of apoptosis and autophagy-related genes were represented statistically as mean ± standard error mean. A paired two-sample Student’s t-test with a p value <0.05 was considered to be statistically significant for expression levels.

3 Results and discussion

3.1 Characterization of AgNPs

Before the intended application for AgNPs in this study has been achieved, the materials were characterized using established techniques. Initially, the color change of the hydrosol to pale brown was taken as an indicator for the synthesis of AgNPs from the precursor silver nitrate by the reductant used [7] (Figure 1). After the initial visual observation was made, DLS, a technique used to measure the nanoparticle size and to analyze their stability for enhanced applications in medicine, was applied [31,32]. Particle size is an important criterion for cellular uptake of nanomaterials inside the tumor and the surrounding environment rich in
capillaries that are 400–600 nm wide. The particle sizes of AgNPs synthesized using varying ratios of the precursor AgNO₃ and the reductant gallic acid (5:5, 8:2 and 9:1) were 148.4, 315.1, and 86.6 nm (Figure 2). These sizes were fewer than 400 nm and therefore can amass in the tumor microenvironment [4,33,34].

After the determination of particle size, an attempt was made to determine the crystalline nature of the synthesized material using XRD at atomic levels. XRD is an influential technique for phase identification, conducting quantifiable investigations and identifying structural differences and particle size of nanomaterials [35–37]. The diffraction pattern illustrates five intense peaks in the entire spectrum at (2θ) 28.23, 32.15, 38.06, 44.43, and 46.57°, which could be linked to (210), (122), (111), (200), and (231) planes that concur to face-centered, cubic, and crystalline silver synthesized using green methods (JCPDS file number: 04-0783). The unassigned peaks are plausibly due to the formation of crystals in the bioorganic phase on the material surface (Figure 3) [38–40]. The crystalline size of the AgNPs calculated using XRD was 38.13 nm. SAED is applied at the nano range to make observations of lattice pattern and crystallinity by use of diffraction spots on a TEM inspecting display from a random particle [41,42]. The SAED pattern of the sample being studied here, revealed bright and sharp rings correlating to (110), (200), (012), (002), (211), and (311) lattice planes of face-centered, cubic, and crystalline silver (Figure 4a) [43–49]. The diffraction patterns observed via

| Gene of interest | Forward primer | Reverse primer |
|------------------|----------------|----------------|
| CASP3            | AGCAAACCTCAGGGAACCATTT | CTGACAGCAGACAAACAAAACCTT |
| CASP8            | GGAGAGGAGTGTGGGGGGA | CGAAGTACGAGGAGGAGGAC |
| CASP9            | AACCTAGAAACCTTACCC | CATCACAAATCTCAGAC |
| CASP12           | GACCAAGCACTGGGATCAA | GCAAGACGCCACATGAGATA |
| Cyt C            | CCAATGAGATGGGAGATG | CGTGAAGCGGAGGAGAC |
| p53              | TGAAGCTCCCAGAATGCCAG | GCTGCCCTG6TGGTTTCT |
| Bax              | GATTGACCGGCTGGGGTCGA | CGGAGGAGTCCAAATGTC |
| Becl-1           | CGGGCGAGACAGATG6GAT | TCTGCCACTATCTTG6GCTT |
| LC3-II           | AGCTCCAAGTGAGCACATTCA | TGTGAGTCTATTTTATG6GCACT |
| CHOP             | GTCTTTTCCAGACTGATCACA | CCTCATACAGGCTCCAGAC |
| ATG3             | GTGAGGAGTGGTCTCTCCG | CGTCAAGCCACACATCTCG |
| ATG12            | CACCAGTACG6CCACAGTAAT | ACTGCAAGCAGGAGAAGTAGA |
| XBP-1            | CTGAGCTCGG6CAACAGGATG | GGCTG6TAAGGAACTG6GTC |
| β-Actin          | ATCTGCGCGTAGCTATTAGGAAGAGAAG | AAGAAAGGAGGCTG6GAAAGTAG |

Figure 1: Observations of initial color in the blank solution and the change in color after incubating gallic acid with solutions containing AgNO₃ at varying concentrations.
both XRD and SAED indicate that the synthesized AgNPs were crystalline in nature and extremely pure. Subsequent to the elucidation of the crystalline nature of the material, TEM, an electron microscopy technique, was adopted to analyze the morphological or structural features of nanomaterials using different magnifications at the atomic resolution [50–52]. The images that are illustrative of the morphology of AgNPs are presented in Figure 4b. The particle size calculated using the data obtained via TEM was 59 nm.

The particle sizes obtained using DLS (the size best suited and used for cytotoxicity studies), XRD, and TEM are less than 100 nm (86.6, 38.13, and 59 nm). The permeable vascular structure of tumors permits therapeutics with high molecular weight and sizes less than 150 nm to extravasate and accumulate into the intratumoral environment around it by enhanced permeability and retention effect [53]. Besides, the absorption, distribution, metabolism, excretion, and biodistribution of particles with sizes greater than 100 nm change drastically. These materials with larger sizes are found in major organs such as spleen, lungs, liver, and kidney over extended periods or durations greater than normal [54]. Hence, particles of sizes

![Differential Intensity (%)](image_url)

**Figure 2:** Analysis of particle size of AgNPs prepared using varying ratios of the precursor and the reductant by adopting DLS.

![XRD pattern](image_url)

**Figure 3:** XRD pattern of the synthesized AgNPs.
less than 100 nm are generally preferred for in vivo applications such as drug delivery [55–57].

The particle sizes of nanomaterials can be obtained using techniques such as DLS, XRD, and TEM [55]. Based on the material to be studied, each technique has its own merits and demerits [58]. Although the particle sizes are less than 100 nm as analyzed using all three methods as evidenced by this study, slight disparities did exist. This disparity was due to the fact that the particle sizes obtained using TEM are generally higher than that of XRD [59,60]. Although the sizes would not change much between TEM and DLS in suspensions with less or no aggregation, samples with agglomerates can give rise to considerably elevated particle sizes using DLS in comparison to TEM [61]. The increased sizes observed in DLS in comparison to that in TEM might be due to the Brownian movement, as DLS measures the Rayleigh scattering from nanoparticles. Considering these differences, DLS is a preferred method for characterizing nanoparticles in aqueous or physiological suspensions proposed to be used for biological applications [35,62].

Gallic acid is an established natural antioxidant and a secondary polyphenolic metabolite distributed, which is available throughout the parts of plants consumed as food, starting from the bark to seed [63,64]. This plant metabolite with reducing properties has the ability to transform metal ions into nanoparticles of metallic forms [65,66]. By virtue of elevated temperatures, the phenolic hydroxyl bonds sustain a homolytic split and produce hydrogen radicals, leading to transfer of electrons from the hydrogen radical to silver ions (Ag+) resulting in the production of AgNPs. This process comprises three stages. In the activation phase, the silver ions are reduced via nucleation resulting in the formation of clusters. Following the first step, the growth phase results in the formation of large-sized particles from relatively small materials by means of spontaneous coalescence designated as Ostwald ripening. In the termination phase, the eventual size of the nanoparticle is reached [67–69].

### 3.2 Cytotoxicity of AgNPs

After characterization of the AgNPs, MTT assay was performed to determine the cytotoxicity of AgNPs on malignant cells, the nanomaterial which is studied and recognized well for such effects [70–72]. This assay is an established preclinical assay to determine the anticancer effect of cytotoxic drugs [73]. The cytotoxicity as a measure of cell viability was dose-dependent (Figure 5). The half-maximal inhibitory concentration (IC50) is a measure of how potent the activity of a tested drug is [74]. The MTT assay determined that the IC50 value for cytotoxic effect of the synthesized AgNPs was 33.45 μg/mL (equivalent to 33.45 ppm), which corresponds well to published reports on

![Figure 4: (a) SAED pattern of the synthesized AgNPs; (b) TEM-based imaging of the morphology of the synthesized AgNPs.](image)

![Figure 5: Assessment of the percentage of cell viability of HT-29 cells using MTT assay after being incubated with varying concentrations of AgNPs.](image)
IC$_{50}$ of AgNPs (3–99 ppm) [75]. The microscopic observations were suggestive of the cytotoxic effect of AgNPs (Figure 6).

According to the outcomes of MTT assay, the activities related to HT-29 cell metabolism were influenced by the AgNPs, and hence, the prospect of induction of apoptosis or autophagy by the materials at nano-regime was evaluated at the IC$_{50}$.

**3.3 Optimization of cell viability by RSM**

The Model F-value (67.24) and the probability value ($p < 0.0001$) for cell viability imply that the model was significant, and the chance of this happening as a result of noise was very low (0.01%) (Table 2). The predicted $R^2$ of 0.9138 was in reasonable agreement with the adjusted $R^2$ of 0.9650; that is, the difference was less than 0.2. The $R^2$ value close to 1 determines that the model appeared significant and fulfilled all requirements of ANOVA. Adeq Precision which measures the signal-to-noise ratio was 23.248, which seems to be adequate. A ratio greater than 4 was found appropriate as observed in this case (Table 3). The resultant response surface and contour plots are presented in Figure 7 [75–78].

**3.4 Analysis of mechanism of cytotoxic effect of AgNPs using quantitative PCR**

AgNPs are considered to be used in the profit-oriented category with the support of numerous toxicological studies [79]. Yet, the ways in which they kill cells derived from mammalian tissues remain less explored. They are known to cause oxidative stress in exposed cells and result in lipid peroxidation, ultimately leading to cell death by mechanisms such as apoptosis, necrosis, or autophagy. When free Ag$^+$ is released into media, they can increase H$_2$O$_2$ levels and result in apoptosis [80,81]. To make a special mention, to provoke cytotoxic effects on mammalian cells, AgNPs are dependent on several parameters including the conditions provided such as duration of exposure; concentration; temperature; and factors such as nanoparticle size, shape, and surface coating in addition to the type of cell being studied [82–84].

Real-time PCR is usually performed to quantify the intracellular mRNA levels and differential gene expression in various cells and tissues [85,86]. It is applied to study the underlying mechanisms of cell death due to the relative advantages they possess in terms of being a sensitive, efficient, accurately quantifying, and high-grade
automated technique [87]. A positive expression fold/ratio determines the upregulation of a gene, while the downregulation of a gene is ascertained by a negative expression fold/ratio [21]. The analysis of gene expression profiles in malignant cells has become an integral part of identifying the biomarkers or changes associated with the disease to improve the chances of personalized therapy. Real-time PCR is therefore considered a standard technique to determine the molecular changes after the cancer cells were exposed to a test drug [88]. Based on this background, real-time PCR was used in this report to study the expression of 14 mRNAs related to autophagy and apoptosis in HT-29 colon cancer cells.

To begin, caspases are proteases which can cleave peptide bonds that follow the aspartic acid residues to initiate and aid in execution of extrinsic apoptosis. Although “isoleucine–glutamic acid–threonine–aspartic acid” is the conventional target motif for CASP8, it can cleave the “aspartic acid–glutamic acid–valine–aspartic acid” target of CASP3. The alteration in expressions of CASP8 may be associated with specific type of tumors. The expressions are usually upregulated in most malignant forms, which can suppress oncogenesis [89–91]. The expressions of CASP8 were upregulated in this report, which is an established initiator of extrinsic apoptosis [92]. CASP12, cleaved by endoplasmic reticulum (ER) stress, can lead to the initiation of intrinsic apoptosis. The upregulation of CASP12 indicates a greater possibility of induction of intrinsic apoptosis [93].

**Table 2: ANOVA and the significance of response surface model for the HT-29 cell viability using CCD**

| Source           | Sum of squares | df  | Mean square | F-value | p-value |
|------------------|----------------|-----|-------------|---------|---------|
| Model            | 9510.32        | 5   | 1902.06     | 67.24   | <0.0001 |
| A – AgNPs (μg/mL)| 13.72          | 1   | 13.72       | 0.4851  | 0.5086  |
| B – Absorbance   | 105.27         | 1   | 105.27      | 3.72    | 0.0951  |
| AB               | 11.78          | 1   | 11.78       | 0.4165  | 0.5392  |
| A²               | 4.25           | 1   | 4.25        | 0.1502  | 0.7098  |
| B²               | 18.58          | 1   | 18.58       | 0.6568  | 0.4444  |
| Residual         | 198.01         | 7   | 28.29       |         |         |
| Cor total        | 9708.33        | 12  |             |         |         |

**Table 3: Regression analysis for the HT-29 cell viability by AgNPs using CCD**

| Std. dev. | R²      | Adjusted R² | Predicted R² | Adeq precision |
|-----------|---------|-------------|--------------|---------------|
| Mean      | 5.32    | 0.9650      | 0.9138       | 23.2477       |
| C.V. %    | 11.66   |             |              |               |

**Figure 7: Response surface and contour plots for the cytotoxicity of AgNPs on HT-29 cells.**
After analysis of the expression of initiator caspases, other mRNAs related to the mechanism of cell-killing were studied. Among these mRNAs studied, p53 plays predominant roles in cell cycle arrest, senescence, and apoptosis. This enables the further existence of damaged cells or eradicates the cells that are critically injured. This determines that this tumor suppressor seems to possess definite functions in non-infectious diseases [94]. To give a special mention, p53 participates directly in the intrinsic apoptosis pathway by regulating the mitochondrial outer membrane permeabilization and producing a trigger in the activity of CASP3 [95]. This mechanism involves cyt-C, Apaf-1, and CASP3 [96]. The contact between apoptotic protease activating factors and mitochondrial cyt-C can activate the caspase cascade, especially CASP3, soon after it enters the cytosol [97,98]. Therefore, the upregulated expressions of p53, cyt-C, and CASP3 indicate the vital role of apoptosis in the observed cell-killing effect.

Bax is a pro-apoptotic target of p53 and has the ability to induce apoptosis in cancer cells [99]. Bcl-2 is predominantly an anti-apoptotic protein and can inhibit both autophagy and apoptosis as cell-killing mechanisms [100]. The upregulated expressions of this protein can result in resistance to intrinsic apoptosis and allow cancer cells to evade apoptosis. Anticancer drugs usually inhibit anti-apoptotic Bcl-2 to induce apoptosis. Yet, the Bcl-2 family of proteins do have both the pro-apoptotic and anti-apoptotic members. Supporting this view, the Bcl-2 family members are classified into three types: anti-apoptotic (Bcl-2, Bcl-xL, Mcl-1, Bcl-w, Bcl-B, and A1/Bfl-1), pro-apoptotic BH3-only proteins (Bim, Bid, Bad, Noxa, Puma, and Bmf), and the multidomains Bax and Bak. The upregulation of pro-apoptotic members of Bcl-2 family by p53 is a common mechanism for apoptosis to occur. The roles of pro-apoptotic effector proteins that comprise BH1, BH2, and BH3 domains and BH3-only proteins (well-known for inhibition of anti-apoptotic Bcl-2 proteins), which might have possibly led to pro-apoptotic Bcl-2 signals, remain critical in the induction of apoptosis along with p53 [101–103]. BH3-only proteins can cause changes in the outer membrane permeability of mitochondria, leading to the release of intracellular cytosolic proteins, which are usually restricted to the inter-membrane space [22]. CHOP is also known to cause an upregulation in expressions of such pro-apoptotic Bcl-2 members leading to elevated production of ROS. This can lead to the release of cyt-C from mitochondria eventually causing an apoptotic trigger [104]. Therefore, the pro-apoptotic members of Bcl-2 might have played crucial roles in the initiation and extension of apoptotic effect and autophagy in the HT-29 cells.

AgNPs have been known to provoke apoptotic cell death in a variety of cancer cells through the induction of ROS and the resultant oxidative stress [105]. After entering the cytosol through the oxidation of cardiolipin

![Graphs showing expression ratios of various genes](image)

**Figure 8:** Fold change over control of apoptotic genes in HT-29 cells using real-time PCR. p values of less than 0.05 (*p < 0.05 and **p < 0.01, compared with the control) are considered significant.
by ROS, cyt-C forms the apoptosome. This is a complex of cyt-C with apoptosis activating factor-1 (Apaf-1), leading to the activation of procaspase-9 which results in activation of CASP9 and an increase in the activity of CASP9 [106,107]. As an initiator and well-characterized caspase with regard to posttranslational modifications, CASP9 which is generally activated by cyt-C release is necessary for the activation of effector CASP3. This is a response to

Figure 9: Fold change over control of autophagy-related genes in HT-29 cells using real-time PCR. p values of less than 0.05 (*p < 0.05 and **p < 0.01, compared with the control) are considered significant.

Figure 10: Possible mechanisms for the cytotoxic effect of AgNPs on HT-29 cells.
death stimuli, the ultimate fate of the cellular apoptosis [108]. CASP8 can cleave another member of the Bcl-2 family, Bid into tBid, which initiates the mitochondrial pathway of apoptosis resulting in the mitochondrial release of cyt-C and Smac/DIABLO. This leads to the formation of apoptosome by interaction of cyt-C with Apaf-1. As a result, the apoptosome complex is formed which activates CASP9. The other mitochondrial protein released, Smac/DIABLO, neutralizes the inhibitory effects of XIAP, leading to apoptotic cell death involving CASP9 and CASP3 [109]. This further elucidates that the mitochondria-dependent apoptosis exerted by AgNPs in HT-29 cells encompasses both extrinsic and intrinsic modes, since the caspase cascade was effectively involved as mentioned previously (Figure 8).

Bclin-1 is a tumor suppressor related to autophagy, which can also mediate apoptosis by interaction with the multidomain proteins of the Bcl-2 family among mammalian cells [110,111]. Autophagy-related (ATG) proteins are crucial members of the canonical pathway of autophagy. In this pathway, the membrane of the endoplasmic reticulum forms the phagophore, which later forms the autophagosome. The autophagosome later fuses with the lysosome to form the autolysosome, which degrades the cargo to be destroyed inside its double-walled membrane, with the support of the ATGs and LC3II (marker of late autophagy) [23,112].

But, the downregulation of ATG12 and ATG3 could be negatively correlated with the formation of ATG12–ATG3 complex, which is necessary to induce autophagy [113]. Therefore, these two significant ATGs did not play a role in the creation of an autophagic flux in HT-29 cells after treatment with AgNPs. Notably, the non-canonical pathway of autophagy does not necessitate the interference of the entire ATGs in the formation of autophagosome, while canonical pathway does. Hence, the elongation and consequent formation of autophagosome membrane might have been dependent on other sources such as WD repeat domain phosphoinositide-interacting protein 1 (WIP1) [114,115]. The outcomes therefore indicate that the autophagy induced in this study was non-canonical.

CHOP is a key transcription factor necessary for the initiation of autophagy [116]. XBP-1 is a key factor in

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**Figure 11:** Schematic representation of the mode of synthesis of AgNPs and the cytotoxicity on HT-29 cells.
unfolded protein response, which is released when a stress such as hypoxia arises in the endoplasmic reticulum. This can induce autophagy in cancer cells via JNK activation and eIF2α phosphorylation [117,118]. Hence, this relates that hypoxia-induced ER stress might have played crucial roles in the observed late autophagy. Autophagy is a stress response and precedes apoptosis. These two mechanisms are concomitant and may arise among cells as a resultant of a stress or an external stimulus [62]. This study determines that these two factors were critical in onset and led to late autophagy via the involvement of LC3II. Therefore, the UPR-activated and CHOP provoked maturation of the autolysosome during AgNPs-induced late autophagy on HT-29 cells was dependent on a non-canonical mechanism (Figure 9).

To conclude, the AgNPs induced mitochondria-dependent apoptosis and late non-canonical autophagy in the carcinoma cells (Figure 10). A crosstalk occurred between autophagy and apoptosis in such cell-killing effect. The schematic representation of the possible mechanism of the synthesis of nanoparticles and the cytotoxicity in HT-29 cells is depicted in Figure 11.

4 Conclusion

In this study, AgNPs were synthesized using gallic acid as a reductant. The synthesis was confirmed initially by the observation of a visible color change after incubating the reductant with the precursor. The synthesized AgNPs were further characterized by established techniques and tested for their cytotoxic effects. The nanoparticles were cytotoxic toward HT-29 cells, and the analysis of mechanisms involved indicated that mitochondria-dependent apoptosis and a late non-canonical autophagy were induced. As a conclusive remark, the present study suggests that AgNPs are valuable candidates for treating cancers of digestive origin. Further preclinical studies are warranted to study the precise effects and promote the clinical use of AgNPs.

Funding information: This study was supported by the National Natural Science Foundation of China (82072704 and 81973525), Jiangsu Primary Research & Development Plan (SBE2021740280), the “333 Talents” Program of Jiangsu Province (BRA2020390), and Project of National Clinical Research Base of Traditional Chinese Medicine in Jiangsu Province (JD2022SZXYA01).

Author contributions: All authors have accepted responsibility for the entire content of this manuscript and approved its submission.

Conflict of interest: The authors state no conflict of interest.

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