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Green tea extract induces protective autophagy in A549 non-small lung cancer cell line*

Ekstrakt zielonej herbaty indukuje autofagię o charakterze protekcyjnym w niedrobnokomórkowym raku płuca linii A549

Authors' Contribution:

- A** Study Design
- B** Data Collection
- C** Statistical Analysis
- D** Data Interpretation
- E** Manuscript Preparation
- F** Literature Search
- G** Funds Collection

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Summary

Background and objectives: For many decades, polyphenols, including green tea extract catechins, have been reported to exert multiple anti-tumor activities. However, to date the mechanisms of their action have not been completely elucidated. Thus, the aim of this study was to assess the effect of green tea extract on non-small lung cancer A549 cells.

Material and methods: A549 cells following treatment with GTE were analyzed using the inverted light and fluorescence microscope. In order to evaluate cell sensitivity and cell death, the MTT assay and Tali image-based cytometer were used, respectively. Ultrastructural alterations were assessed using a transmission electron microscope.

Results: The obtained data suggested that GTE, even at the highest dose employed (150 µM), was not toxic to A549 cells. Likewise, the treatment with GTE resulted in only a very small dose-dependent increase in the population of apoptotic cells. However, enhanced accumulation of vacuole-like structures in response to GTE was seen at the light and electron microscopic level. Furthermore, an increase in the acidic vesicular organelles and LC3-II puncta formation was observed under the fluorescence microscope, following GTE treatment. The analysis of the functional status of autophagy revealed that GTE-induced autophagy may provide self-protection against its own cytotoxicity, since we observed that the blockage of autophagy by bafilomycin A1 decreased the viability of A549 cells and potentiated necrotic cell death induction in response to GTE treatment.

Conclusion: Collectively, our results revealed that A549 cells are insensitive to both low and high concentrations of the green tea extract, probably due to the induction of cytoprotective autophagy. These data suggest that a potential utility of GTE in lung cancer therapy may lie in its synergistic combinations with drugs or small molecules that target autophagy, rather than in monotherapy.

Key words: green tea extract • autophagy • non-small lung cancer

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INTRODUCTION

Tea is one of the most popular beverages in the world. All tea is produced from the leaves of *Camellia sinensis*, but the type of tea depends on the process of their manufacture [32]. All types of tea contain active polyphenols, commonly known as catechins [1]. Green tea contains polyphenols such as (-)-epigallocatechin, (-)-epigallocatechin-3-gallate (EGCG), (-)-epicatechin, and (-)-epicatechin-3-gallate, but EGCG is the most abundant and possibly the most bioactive [28]. Many epidemiological studies have shown that green tea may help protect against cancer development [32]. It is possible that EGCG acts proactively against esophageal, lung, prostate, stomach, intestine, breast and colon carcinogenesis [5,12,22,23,32]. Green tea extract (GTE) consumption is also believed to protect against the development of atherosclerosis and coronary heart disease, high blood cholesterol concentrations, and high blood pressure [19]. It is known that the biological activities of EGCG are associated with anti-inflammatory and anti-oxidant effects [9]. Furthermore, the study on the mechanisms involved in chemoprevention of green tea has revealed its impact on the modulation of signal transduction pathways that lead to the inhibition of cell proliferation and transformation, inhibition of tumor invasion and angiogenesis and induction of cell death [15]. The most common types of cell death which are induced by EGCG are apoptosis and autophagy. Additionally, the crosstalk between autophagy and apoptosis has recently been described [7]. The first type of cell death is characterized by chromatin condensation, nuclear fragmentation and blebbing formation. EGCG induces apoptosis and inhibits proliferation in numerous cancer cell lines, inter alia in gastric cancer cells and human breast cancer cells [17,27]. It has also been shown that GTE enhances the cytostatic effect of conventional anticancer drugs in chemoresistant cancer cells. Chen et al. (2013) reported that the combination of EGCG and sulforaphane induces apoptosis in paclitaxel-resistant ovarian cancer cells. Likewise, Du et al. showed the synergistic apoptotic effect of panaxadiol and EGCG in human colorectal cancer cells [3,6]. Autophagy, the second type of cell death that has been observed in cells treated with GTE, is characterized by the formation of autophagosomes and autophagolysosomes containing unnecessary or dysfunctional cellular components and unused proteins

[31]. In the literature, three types of autophagy are described – microautophagy, chaperone-mediated autophagy and macroautophagy – but the best described and understood is macroautophagy. Autophagy can be stimulated by lack of nutrient, reactive oxygen species, hypoxia and toll-like receptor agonists. It is known to be involved in inflammation, aging, cancer, neurodegeneration, cardiac myopathies and infections [30]. Generally, autophagy inhibits apoptosis, but in special cases, autophagy can induce apoptosis. Additionally, massive autophagy has been shown to promote autophagic cell death, also known as type II programmed cell death [18].

The aim of the present study was to determine the effect of GTE on human non-small cell lung carcinoma cells (A549).

MATERIAL AND METHODS

Cell culture and treatment

A549 cells (non-small lung cancer cell line; NSCLC) were grown in a monolayer at 37°C in a humidified CO₂ incubator (5% CO₂) in Dulbecco's Modified Eagle's Medium (DMEM, Lonza) with the addition of 10% FBS (fetal bovine serum; Gibco) and 50 µg/ml of gentamycin (Sigma-Aldrich). A stock solution of green tea extract (GTE, Santa Cruz) was prepared in distilled water and stored at low temperatures until use. The required concentrations of GTE (25, 50, 100, 150 µM) were added to cells for 24 h for MTT assay. For other experiments the non-small lung cancer cells were treated with GTE at final concentrations of 25, 50 and 150 µM for 24 h. Control cells were incubated under identical conditions without the addition of green tea extract. Furthermore, in order to inhibit autophagy the cells were pretreated for 8 h with bafilomycin A1 (Baf A1) at a concentration of 100 nM followed by 24 h incubation with GTE. The A549 cells were observed using an inverted microscope (Nikon).

MTT assay

The cytotoxicity of GTE was assessed using the MTT assay. Viable cells have the ability to reduce the yellow methyl thiazolyl tetrazolium (MTT) to purple formazan crystals by mitochondrial dehydrogenase enzymes. The A549 cells were

harvested and seeded on 12-well culture plates. A MTT stock solution was prepared fresh as 5 mg/ml in PBS and filtered through a 0.22µm filter. After 24 h of culture with GTE the MTT solution was mixed with DMEM without phenol red in the ratio 1:9, added to each culture well and incubated in the dark for 3 h at 37°C. Then, the formazan crystals were dissolved in isopropanol and were centrifuged at 13 000 RPMI for 2 min. The absorbance of the resulting purple solution was spectrophotometrically measured at a wavelength of 570 nm (Spectra Academy, K-MAC, Korea). Each experiment was repeated three times. The viability of the non-small cancer cell line was calculated as the percentage of MTT reduction and the absorbance of control cells was assumed as 100%.

Cell death analysis by annexin V/PI assay

Cell death was assessed by double staining with annexin V and propidium iodide (Tali Apoptosis Assay Kit – Annexin V Alexa Fluor 488 and Propidium Iodide, Invitrogen). Annexin V is widely used to identify early apoptotic cells, while propidium iodide is indicative of necrosis. Late apoptotic cells are characterized by a positive signal for both annexin V and PI. The procedure was performed in accordance with Invitrogen's protocol. The A549 cells were harvested on 6-well plates by trypsinization and centrifuged (1800g, 8 min). Next, the cells were incubated with Annexin V Alexa Fluor 488 for 20 min in the dark (room temperature). Later, after centrifugation (300g, 5 min), propidium iodide was added for 5 min in the dark (room temperature). Cell death was analyzed using the Tali Image-Based Cytometer (Invitrogen).

Statistical analysis

The nonparametric Mann-Whitney U test was used for statistical analysis of differences between doses of GTE. The results were considered significant at $p < 0.05^*$. Statistical analysis was carried out with GraphPad Prism (version 5.0; GraphPad Software).

Acridine orange staining

One method for detecting autophagy is acridine orange staining (AO). This weak base is used for detection of acidic vesicular organelles (AVOs) and as a lysosomal dye. For the experiment, the A549 cells were grown on coverslips. The acridine orange stock solution was prepared fresh as 1 µg/ml in PBS and was added to the culture medium to each well of the plate and incubated for 20 min at 37°C. After removal of the medium, cells were washed with PBS (2 x 5 min) and coverslips were mounted with PBS. The fluorescence of orange acridine was examined using a Nikon Eclipse E800 fluorescence microscope (Nikon; Tokyo, Japan) and NIS-Elements 4.0 software (Nikon).

Immunofluorescent labeling of LC3-II

To examine the level and pattern of LC3-II immunostaining, the non-small cell lung cancer cells were grown on coverslips and fixed with 4% paraformaldehyde for

20 min at room temperature and washed with PBS (3 x 5 min). In the next step, 0.25% Triton X-100 was added (5 min, room temperature [RT]). Then the cells were rinsed with PBS (3 x 5 min, RT) and incubated with 1% BSA. LC3-II staining was performed using a mouse monoclonal antibody specific for LC3-II for 1 h (diluted 1:1000 in BSA). After washing with PBS (3x 5 min), cells were incubated with goat anti-mouse secondary antibody diluted in PBS 1:100 for 1 h. The cell nuclei were labeled using DAPI for 10 min (diluted 1:20 000 in PBS). Finally, the coverslips were mounted with PolyAqua/Mount and were analyzed with a Nikon Eclipse E800 fluorescence microscope (Nikon; Tokyo, Japan) and NIS-Elements 4.0 software (Nikon).

Transmission electron microscopy (TEM)

The next step was the observation of the morphological changes at the ultrastructural level. For this purpose transmission electron microscopy was used. The A549 cells were grown on 6-well plates and harvested with trypsin. Then cells were fixed with 3.6% (v/v) glutaraldehyde in 0.1 M sodium cacodylate buffer for 30 min at room temperature, and three times washed with 0.1 M sodium cacodylate buffer. Bovine thrombin and fibrinogen were used to form fibrin clots with cells entrapped. The cells were post-fixed with 2% (w/v) OsO₄ in 0.1 M sodium cacodylate buffer (1 h, RT), and next rinsed with 0.1 M sodium cacodylate buffer, dehydrated through a graded ethanol (30–90%) and acetone (90–100%) series. Afterwards, the cells were embedded in a mixture of Epon 812, cut into ultrathin sections using an OmU3 ultramicrotome (Reichert) and placed on copper grids. Then, the ultrathin sections were stained with 1% uranyl acetate and lead citrate. The preparations were examined using the JEM 100 CX electron microscope (Jeol).

RESULTS AND DISCUSSION

We first assessed the sensitivity of the A549 non-small lung cancer cell line to the green tea extract. For this purpose, we treated A549 cells with GTE at concentrations of 25, 50 and 150 µM for 24 h and MTT colorimetric assays were performed. The obtained data suggested that GTE, even at the highest dose, was not toxic to A549 cells. As shown in Figure 1, compared to the control populations, 97.06, 94.31 and 93.56% of A549 cells survived after exposure to 25, 50 and 150 µM GTE, respectively. Next, we performed apoptosis analysis of A549 cells in response to GTE treatment. The cells were double-stained with Annexin V and PI and the percentage of cells in each population (viable Annexin V-/PI-; early apoptotic Annexin V+/PI-; late apoptotic Annexin V+/PI+; necrotic Annexin V-/PI+) was evaluated using an image-based cytometer. The sum of the early and late apoptotic cells represented the total apoptosis. As can be seen in Figure 2B, the treatment with 25, 50 and 150µM GTE for 24 h resulted in a small but statistically significant increase in the population of apoptotic cells from 0.075% to 0.45-3.2%. Moreover, there were no statistically significant differences in the percentage of ne-

croctic cells after the exposure of A549 cells to green tea extract when compared to the control, with the exception of the highest dose of GTE plus Baf A1 and Baf A1 alone (Fig. 2C, see the text below).

To study the effect of green tea extract on A549 cells we used an inverted microscope to analyze the cell morphology. After the treatment with GTE, a lot of vacuole-like structures in the cytoplasm of A549 cells were seen (Fig. 3). The severity of these changes appeared to be directly related to the GTE doses. To make sure that the observed vacuole-like structures were in fact due to the induction of autophagy, we used a specific autophagy inhibitor, bafilomycin A1, which prevents the maturation of autophagic vacuoles by inhibiting the fusion between autophagosomes and lysosomes [13,29]. Baf A1 pretreatment almost completely abolished GTE-induced vacuole-like structure

formation in A549 cells, confirming that those changes were associated with autophagy. This finding, together with the observation of high viability of A549 cells after treatment with GTE, led us to presume that GTE might induce autophagy in these cells, because many previous studies have shown that autophagy i) is associated with the formation of autophagosomes and autophagolysosomes and ii) facilitates the resistance of tumor cells against chemotherapy and radiation [4,20]. To further confirm whether GTE could trigger autophagy, we studied the ultrastructure of GTE-treated A549 cells in the transmission electron microscope, since TEM has been considered as a gold standard to observe autophagic vacuoles [14]. As we expected, GTE promoted the accumulation of autophagic vacuoles, which exhibited autolysosomal and/or autophagosome characteristics (Fig. 4). Numerous empty vacuoles as well as vacuoles filled with remnants of organelles were observed (Fig. 4C,D). Another characteristic ultrastructural feature of autophagy was noticed: swollen mitochondria devoid of cristae.

To further study the incidence of autophagy, we examined the impact of GTE on the occurrence of acidic vesicular organelles (AVOs) by staining cancer cells with acridine orange (AO). The detection of AVOs, which include autophagic vacuoles and lysosomes, is a standard method for monitoring autophagy [21]. AO is a fluorescent weak base that accumulates in acidic spaces, such as AVOs, and emits bright red fluorescence. The intensity of the red fluorescence is therefore proportional to the degree of the acidity and the volume of acidic vesicular organelles [2]. As shown in Figure 5, in A549 cells GTE promoted the formation of AVOs in a dose-dependent manner, the process of which was almost completely blocked by pretreating with Baf A1. We also examined the effect of GTE on the intensity and pattern of LC3-II (a specific marker of autophagy) immunostaining. The fluorescence microscopy analysis

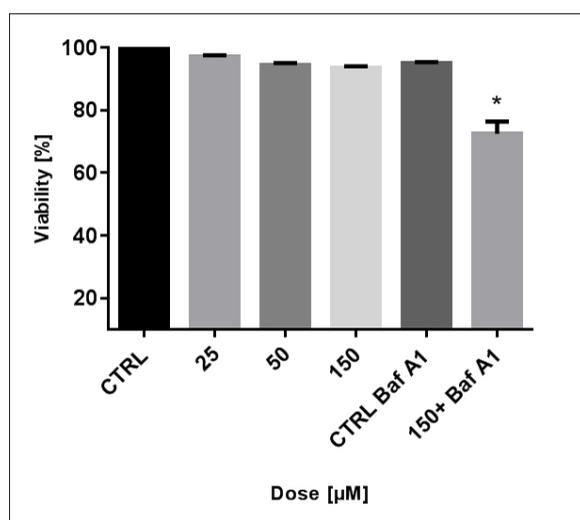


Fig. 1. MTT assay. Cell viability was measured with an MTT assay. Data represent means of three independent experiments

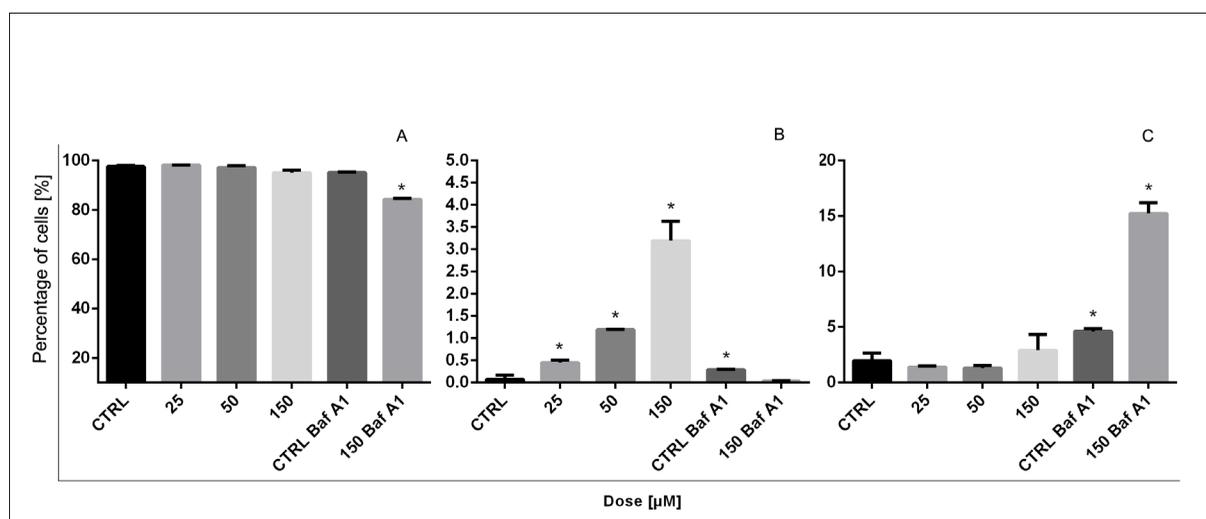


Fig. 2. Effect of GTE on induction of cell death. A549 cells were treated with 25, 50, 150 μ M GTE for 24 h and double-stained with Annexin V Alexa Fluor 488 and Propidium Iodide and analysis was performed using an image-based cytometer. CTRL – control. Percentage of live cells (A); Percentage of apoptotic cells (B); Percentage of necrotic cells (C)

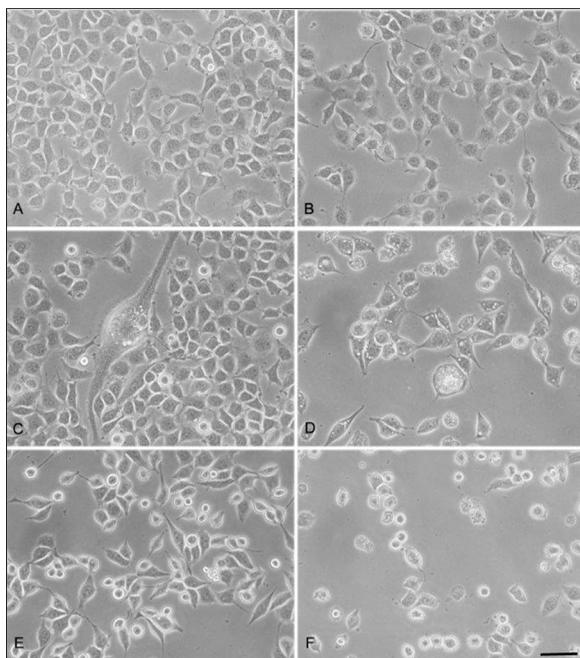


Fig. 3. Effect of GTE evaluated by inverted microscope. A549 cells were treated with 25, 50, 150 μ M GTE for 24 h. Control A549 cells (A); A549 cells treated with 25 μ M GTE (B); A549 cells treated with 50 μ M GTE (C); A549 cells treated with 150 μ M GTE (D); A549 cells treated with 100 nM Baf A1 (E); A549 cells treated with 150 μ M GTE and 100 nM Baf A1 (F). Bar = 50 μ m

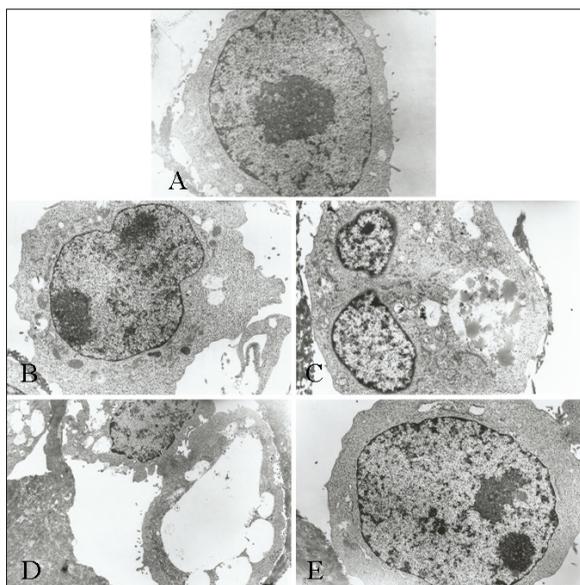


Fig. 4. Effect of GTE at the ultrastructural level. A549 cells were treated with 25, 50, 150 μ M GTE for 24 h and examined by transmission electron microscopy. Control A549 cells, x 5000 (A); A549 cells treated with 25 μ M GTE, x 5000 (B); A549 cells treated with 50 μ M GTE, x 5000 (C); A549 cells treated with 150 μ M GTE, x 3300 (D); A549 cells treated with 150 μ M GTE and 100 nM Baf A1, x 5000 (E)

revealed the punctate staining pattern and the increased fluorescence intensity of this protein in the A549 cells exposed to GTE (Figure 6), in comparison to the

diffuse staining pattern and low fluorescence intensity of LC3-II in control cells. Such an effect of GTE was dose-dependent. Pretreatment with Baf A1 resulted in further accumulation of LC3-II in A549 cells (Fig. 6E). Collectively, our results suggested that GTE, especially at higher doses, triggered autophagy in A549 cells. Similar observations have been presented by Kim et al. (2013), who observed an increase in the formation of LC3-II and autophagosomes following treatment with EGCG of primary bovine aortic endothelial cells (BAEC) [10]. Several other studies have shown that the most important component of GTE, - (-)-epigallocatechin-3-gallate (EGCG), may induce autophagy. Also, Kim et al. (2013) demonstrated that EGCG stimulates autophagy in vascular endothelial cells [10].

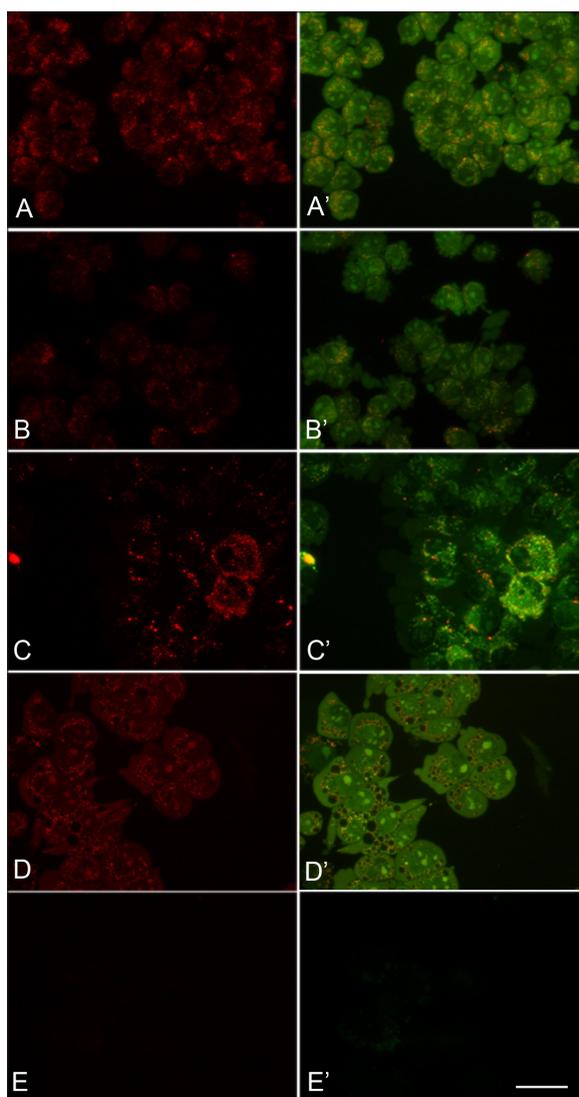


Fig. 5. Effect of GTE on induction of AVOs. A549 cells were treated with 25, 50, 150 μ M GTE for 24 h and examined by classical fluorescence microscope. Control A549 cells (A,A'); A549 cells treated with 25 μ M GTE (B,B'); A549 cells treated with 50 μ M GTE (C,C'); A549 cells treated with 150 μ M GTE (D,D'); A549 cells treated with 150 μ M GTE and 100 nM Baf A1 (E,E'). Bar = 50 μ m

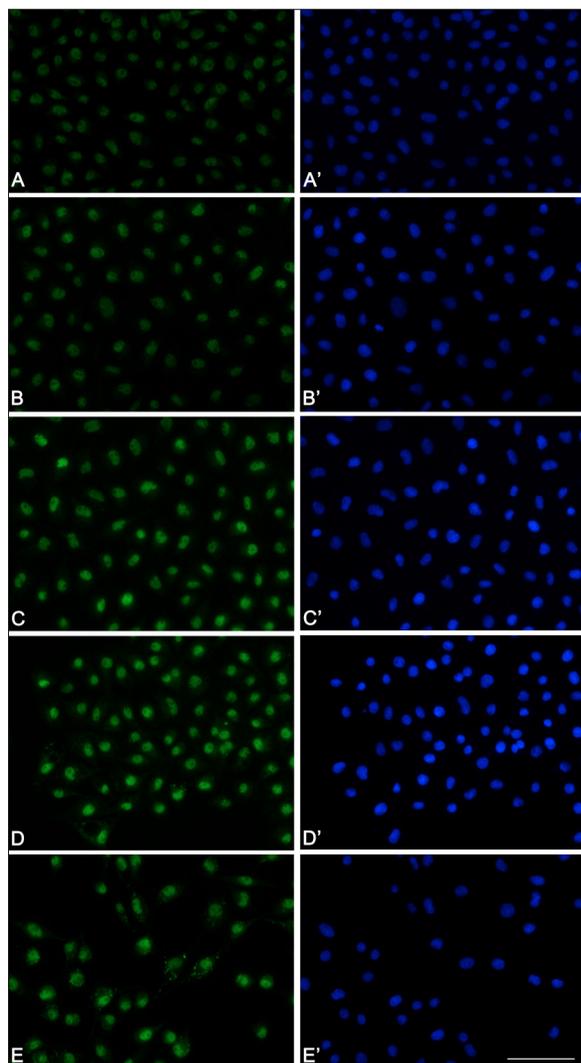


Fig. 6. Effect of GTE on induction of LC3II. A549 cells were treated with 25, 50, 150 μM GTE for 24 h and examined by classical fluorescence microscope. Control A549 cells (A,A'); A549 cells treated with 25 μM GTE (B,B'); A549 cells treated with 50 μM GTE (C,C'); A549 cells treated with 150 μM GTE (D,D'); A549 cells treated with 150 μM GTE and 100 nM Baf A1 (E,E'). Bar = 50 μm

As mentioned above, the high viability of A549 cells after treatment with GTE allowed us to assume that there must be some mechanism that protects these cells against GTE-induced cytotoxicity. Even though autophagy has recently been proposed to be a type of cell death, the primary role of

autophagy is to protect cells under stressful conditions such as starvation, hypoxia and drug treatment as well [16]. For example, Satoh et al. (2013) studied the effect of EGCG on five human mesothelioma cell lines and revealed that the inhibition of autophagy by chloroquine (CQ) enhanced the EGCG-induced cell death [24]. Therefore, we presumed that GTE-induced autophagy may have a cytoprotective effect on A549 cells. To test this hypothesis, we pretreated A549 cells with Baf A1 for 8 h and then incubated them for 24 h with GTE. After that, the MTT assays were performed. As shown in Figure 1, autophagy blockage significantly decreased the viability of A549 cells from 95.10% (Baf A1 alone) to 72.43% (Baf A1 plus 150 μM GTE), confirming our assumptions. Since it has been shown that the inhibition of the cytoprotective role of autophagy may enhance the apoptotic response to anticancer drugs [26], we repeated the Annexin V/PI assays using Baf A1. Surprisingly, we did not observe an increase in the percentage of apoptotic cells in the populations co-treated with Baf A1 and GTE at a concentration of 150 μM in comparison to cells exposed to Baf A1 alone (Fig. 2B). Therefore, we assume that autophagy and apoptosis in A549 cells treated with GTE are independent processes. Indeed, there is currently accumulating evidence that autophagy and apoptosis can act either as partners to induce cell death or autophagy may act as an antagonist to inhibit apoptosis, thereby promoting cell survival, or apoptosis and autophagy may occur independently of each other [8,11]. However, the blockade of autophagy by Baf A1 resulted in increased necrotic cell death in response to GTE (from 1.98% and 4.62% in the control and Baf A1-treated cells, respectively, to 15.25% in co-treated cells), further suggesting that GTE-induced autophagy plays a cytoprotective role against its own cytotoxic effect.

To sum up, the results presented here revealed that A549 cells are insensitive to both low and high concentrations of the green tea extract, probably due to the induction of cytoprotective autophagy. These data confirm a common observation that dietary polyphenols alone fail to affect the growth of chemoresistant cancer cells. Therefore the potential of these compounds as anticancer agents lies in combination therapies with conventional cytostatic drugs to decrease their effective doses and/or enhance their efficacy. It is important to clarify, *in vitro* and *in vivo*, whether autophagy inhibitors such as chloroquine, which is currently under clinical trials, could act synergistically with GTE to produce a growth inhibitory effect on chemoresistant cancer cells [11,25].

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