Research Article

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Induction of apoptosis and autophagy *via* regulation of AKT and JNK mitogen-activated protein kinase pathways in breast cancer cell lines exposed to gold nanoparticles loaded with TNF-α and combined with doxorubicin

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Abstract: Gold nanoparticles (GNPs) tagged with peptides are pioneers in bioengineered cancer therapy. The aim of the current work was to elucidate the potential anticancer interactions between doxorubicin and GNPs loaded with tumor necrosis factor-alpha (TNF-α). To investigate whether GNPs loaded with TNF and doxorubicin could stimulate autophagy and apoptosis in breast cancer cells. Two human breast cancer cell lines, MCF-7 and AMJ-13, as well as different apoptotic and autophagy markers, were used. In both cell types, treatment with TNF-loaded GNPs in conjunction with doxorubicin increased the production of apoptotic proteins including Bad, caspase-3, caspase-7, and p53 with upregulation of the LC3-II and Beclin1 proteins. In addition, the findings showed that the mitogen-activated protein kinase signaling pathway was dramatically affected by the GNPs loaded with TNF-α and combined with doxorubicin. This had the effect of decreasing p-AKT while simultaneously increasing p-JNK1/2. The findings demonstrated that GNPs loaded with TNF-α and combined with doxorubicin can induce both autophagy and apoptosis in breast cancer cells. These results suggest that TNF- and doxorubicin-loaded GNPs provide a therapeutic option as a nanomedicine to inhibit the proliferation of breast cancer.

Keywords: GNPs, TNF-α, doxorubicin, breast cell line, cytotoxicity, apoptosis, autophagy

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1 Introduction

Nanotechnology is a wide and rapidly expanding field that involves the production of organic and inorganic materials as well as their conversion and molecular level manipulation, producing materials with particular biological, chemical, and physical properties, it is an exciting new science that has the potential to change a broad number of sectors, including the treatment of cancer [1,2]. Nanomaterials are substances with unique optical, magnetic, and electrical properties. They are materials between 1 and 100 nm in size [3–5].

A variety of nanomaterials were discovered to accumulate within autophagosomes and even to enhance autophagosome formation. Quantum dots were found to have an inducing influence on autophagy, which was initially documented by Seleverstov *et al.* [6]. Several types of nanomaterials such as silica, gold, and alumina were found to accumulate within autophagosomes [6]. Aggregated nanoparticles enter into cells by endocytosis much more than well-dispersed nanoparticles. The internalized nanoparticles may be regarded as foreign materials and autophagic cargos by cells and then trigger autophagy [7,8].

Cancer is a disorder characterized by uncontrolled cell differentiation, which has been managed using a number of approaches, including chemotherapy, radiation therapy, and the surgical removal of affected tissue [9]. Although each of these treatments appears to be successful regarding the death of cells, they all have severe and nonselective adverse effects on patients. Recently, there has been a lot of interest shown in cancer therapy involving nanomedicine-mediated modalities due to their active/passive targeting, high solubility/bioavailability, biocompatibility, and multi-functionality. This is to overcome the side effects that are associated with traditional cancer treatments [10].

Gold nanoparticles (GNPs) were shown to have a greater anti-tumor and anti-proliferative effect against cancer cells due to the generation of reactive oxygen species (ROS), which cause oxidative stress and ultimately result in the death of the cells [11]. ROS are generated in the mitochondria of a cell. ROS causes severe damage to cellular macromolecules, particularly DNA. The level of DNA damage that a cell sustains either stops the cell cycle, causes DNA repair to take place, or activates the pathways that lead to apoptosis [12]. As a consequence of this, ROS play a prominent and critical role in both apoptosis and autophagy, which are two processes that ultimately lead to the death of cells [13]. The term programmed cell death is more commonly used to refer to apoptosis that causes minimal damage to surrounding cells and tissues and does not cause inflammation. This is in contrast to necrosis, which results in severe damage to cell organelles and a loss of the integrity of the cell membrane. Apoptosis also causes less damage than necrosis does [14].

A previous study focused on the important role of autophagy as a critical mechanism in nanoparticle-induced toxicity and the physicochemical and biochemical mechanisms of autophagy triggered by nanoparticles [15]. Interestingly, nanomaterials can modulate autophagy and serves as a double-edged sword, that could be utilized in the treatment of certain diseases related to autophagy dysfunction, such as cancer, neurodegenerative disease, and cardiovascular disease [16]. This finding is consistent with the hypothesis that autophagy is a mechanism that reduces the number of mitochondria that are damaged. This could result in the formation of nanoparticles. Furthermore, regarding autophagy induction, nanoparticles can cause lysosomal dysfunction. In the previously published studies, it was found that exposure to nanomaterials causes lysosomal dysfunction [17,18]. Autophagy is a process that occurs in eukaryotic cells. It is an essential homeostasis and cell survival process that reacts to environmental challenges such as hunger or infection from pathogens [19]. Newly and recently collected data suggest that autophagy may also occur under pathological processes [20], such as in the development of neurological diseases or tumors. In particular, the process of autophagy is thought to play a significant part in the growth of tumors [21]. Autophagy serves as a tumor suppressor in the early stages of tumor formation; nevertheless, autophagic activity is frequently reduced in cancer cells [22].

Beclin 1 is a crucial protein in the process of phagophore production, which suggests the idea that Beclin 1 plays a role as a tumor suppressor. Further studies indicated that the BECN1 gene functions as a tumor suppressor, which was provided by findings showing that the absence of BECN1 in cancer cell lines and mouse models leads to an increase in cell proliferation and a decrease in autophagy [23,24]. In addition, a number of studies [25,26] demonstrated that malignancies of various types, such as cervical squamous cell carcinomas and hepatocellular carcinomas, have lower levels of the protein known as Beclin 1. According to the findings of a previous study, inhibiting essential genes involved in autophagy can reduce the growth of tumors in patients with cancer. A number of proteins, including UV radiation resistance-associated genes (UVRAG) and Bax-interacting factor-1 (Bif-1), which associate with BECN1, operate as tumor suppressors and positively regulate autophagy [27]. The damage of autophagosome formation and autophagy led to an increase in the proliferation of cancer cells in colon, stomach, breast, and prostate cell lines [28–30]. This was caused by UVRAG being depleted and Bif-1 being reduced. In mice with a knockout of the autophagic essential proteins,

the deletion of ATG5 and ATG7 causes liver cancer to develop in autophagy-deficient hepatocytes. This is because damaged mitochondria and oxidative stress contribute to the development of liver cancer [31]. Additional investigations demonstrated that a lack of autophagic regulators such as ATG3, ATG5, and ATG9 is connected with the development of cancer [32,33]. When these mice were subjected to chemical carcinogens, researchers found that they were more likely to develop fibrosarcomas [34]. This increased susceptibility was seen in mice that lacked the ATG4 gene. In addition to this, autophagy inhibits the development of tumors by controlling the production of ROS. When mitochondria are damaged, an excessive amount of ROS is produced, which ultimately leads to the development of carcinogenesis [35–37]. According to these findings, autophagy is likely an essential process that prevents the formation of tumors, and dysfunction in autophagy may lead to the development of ontogenesis. Consistent with the findings of the current study, GNPs loaded with tumor necrosis factor-alpha (TNF-α) and combined with doxorubicin have the ability to modulate the protein kinase B (AKT) and Jun NH2-terminal kinase (JNK) pathways in breast cancer cells, by inducing autophagy and apoptosis.

2 Materials and methods

2.1 2,5-diphenyl-2H-tetrazolium bromide (MTT) assay

The cytotoxicity of GNPs loaded with TNF-α and doxorubicin was measured by MTT assay. Human breast cancer cell line (MCF-7), Iraqi female breast cancer cell line (AMJ-13), and normal Rat Embryonic Fibroblasts cell line (REF) were seeded into 96-well plates at a density of 1×10^4 cells/well. The cell lines were kindly provided by the Iraqi Center for Cancer and Medical Genetic Research, AL-Mustansiriya University, Baghdad, Iraq. Different concentrations (3.1, 6.25, 12.5, 25, and 50 µg/mL) of GNPs loaded with TNF-α alone, doxorubicin alone, and both as combined therapy were added to the cells for a period of 48 h, followed by a wash with PBS. MTT solution was added to the cells at a concentration of 2 mg/mL (Invitrogen, Carlsbad, CA) for 3 h. After that, the solution was drained out of each well, and then, 100 µL of DMSO was added to each sample. Spectrophotometers were used to determine each sample's absorbance at a wavelength of 492 nm [38]. The equation that was used to determine the percentage of cytotoxicity is as follows.

$$\text{Cytotoxicity\%} = \frac{A - B}{A},$$

where A represents the optical density of the control and B represents the optical density of the samples [39].

2.2 Colony-forming assay

At a density of 10,000 cells/mL, breast cancer cells were seeded onto 24-well plates. After waiting 24 h, the cells were treated with GNPs loaded with TNF-α and doxorubicin at IC_{50} concentration. When the cells attained monolayer confluence, cells were washed three times using sterile PBS. The colonies of breast cancer cells were fixed using absolute methanol. After that, they were stained using crystal violet (Sigma-Aldrich, USA) for 15 min. The samples were washed with DW to remove any excess stain [40].

2.3 Acridine orange/ethidium bromide staining (AO/EtBr)

In 98-well plates, the MCF-7, AMJ-13, and REF cells were seeded at a concentration of 10,000 cells/well. Following a 24-h incubation period, the cells were exposed to GNPs loaded with TNF-α and doxorubicin alone and combined at IC_{50} concentration for 24 h. Following that, the cells were stained with 1 µg/mL AO/EtBr for 2 min at 37°C and examined by fluorescent microscope [41].

2.4 Proteomic profile array

This assay was completed, in accordance with the protocol of the manufacturer, to estimate the pathway of apoptosis resulting from cancer cell lysate after being treated with GNPs loaded with TNF-α alone and doxorubicin alone and both as a combined therapy. Human Apoptosis Antibody Array C1 Kit (RayBio) was used to measure which proteins were causing apoptosis. The cell lysate was harvested, and then, the extracted proteins were calculated by nanodrop (Thermofisher, USA) and normalized. The antibody array against human apoptosis was incubated overnight with 250 µg of proteins extracted from treated and untreated cells. A Biospectrum AC ChemiHR device was used to scan the membranes that were utilized in the process of quantifying the apoptosis array data. ImageJ was used to conduct the statistical analysis, and the signal fold

expression levels of each specimen were calculated in accordance with the guidelines of the manufacturer.

2.5 Flow cytometry assay

For the purpose of measuring the LC3 marker, erk1,2 marker, and Mitochondrial Membrane potential (MMP) using (JC-1) probe in the breast cancer cell lines after being treated with GNPs loaded with TNF-α alone, doxorubicin alone, and both as a combined therapy, a flow cytometry test was completed in accordance with the protocol of the manufacturer. The fluorescence intensity of the cells was measured using a flow cytometer.

2.6 Immunofluorescence assay

MCF-7 and AMJ-13 cells were plated at 10^5/well in an 8-well chamber slide. The cells were treated with GNPs loaded with TNF-α, doxorubicin alone, and both combined at concentration IC_{50} for 8 h. After that, the cells were fixed by 4% PFA for 30 min. Then, cells were washed two times with PBS and then permeabilized using SDS 0.1% for 10 min. The samples were blocked by 10% fetal calf serum for 60 min at room temperature. Then, cells were incubated (2 h at room temperature) with primary antibodies (anti-JIK, Cat. No. ab4821, anti-AKT, Cat. No. ab235958, anti-cleaved caspase-3, Cat. No. ab32042, anti-LC3, Cat. No. ab48394, and anti-Beclin 1, Cat. No. ab62557, were purchased from Abcam). After that, cells were washed four times using 1× PBS. The secondary antibodies (Alexa fluor 488, Cat. No. ab150177, and Alexa fluor 568, Cat. No. ab175712 were purchased from Abcam) were added to the cells and incubated for 2 h at room temperature. Finally, five times washing of the cells in 1× PBS was conducted. Fluorescent images were captured using a confocal microscope [42].

2.7 Statistical analysis

The data of three independent experiments are represented as mean ± standard deviation. GraphPad Prism (7) was used to carry out the statistical analysis *via* the application of the one-way ANOVA analysis of variance. The difference between means was assessed by LSD, in which $p \leq 0.05$ was considered significant. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, ****$p \leq 0.0001$.

3 Results and discussion

3.1 GNPs loaded with TNF-α and doxorubicin inhibit breast cancer cell lines' proliferation

After 48 h of treatment with various concentrations of the tested materials (3.1, 6.25, 12.5, 25, and 50 µg/mL), the cytotoxicity percentage and inhibit growth were measured. GNPs loaded with TNF-α, doxorubicin alone, and both combined suppressed cell viability in a concentration-dependent manner, as shown in Figures 1 and 2. The IC_{50} for all tested compounds is represented in Figures 1 and 2 (right panels). Figure 3 represents the anti-proliferative activity of the GNPs loaded with TNF-α, doxorubicin alone, and both combined on REF cells as a normal cell line. MET and the chemotherapeutic medication doxorubicin (DOX) were loaded onto size-shrinkable RGD-DGL-GNP nanoparticles (RDG NPs) for the purpose of combination therapy. pH-sensitive imine bonds were used in the loading process. The RGD-MET-DGL-GNP nanoparticles (RMDG NPs) were able to deeply penetrate the tumor to deliver MET and inhibit NF-ƙB activity in tumor cells, leading to a reduction in the expression of tumor necrosis factor (TNF-α) and interleukin-6 in tumor tissues as well as a suppression of the proliferation of tumor cells. In a xenograft tumor model and lipopolysaccharide-induced pulmonary metastasis model with murine 4T1 breast cancer and CT26 colon cancer cells, the co-administration of RGD-DOX-DGL-GNP (RDDG NPs) and RMDG NPs induced an improved therapeutic effect. The considered strategy is a very successful anti-cancer technique, combining RDDG and RMDG NPs to simultaneously target tumors as well as inflammation that is associated with cancer [43]. Gallic acid (GA) was delivered to cancer cells by spherical GNPs with a size of 15 nm, and this was accomplished so that the anti-cancer action may be more effective. The capacity of GNPs–GA complex to prevent the development of cervical cancer cells is diminished when compared to that of unmodified GA. It is interesting to note that normal cells can tolerate large concentrations (150 M) of GNPs–GA without suffering damage, whereas GA alone is cytotoxic. In a nutshell, GNPs–GA is not as effective as GA in preventing the proliferation of cervical cancer cells, but it does not harm normal cells and is not cytotoxic. As a result, GNPs could be employed as an alternative phytochemical delivery method for the treatment of cancer [44,45]. This would lessen the negative effects that are associated with radiotherapy and chemotherapy. Nevertheless, nano-gold loaded with resveratrol (Res-GNPs, 39 nm) exhibits better anti-

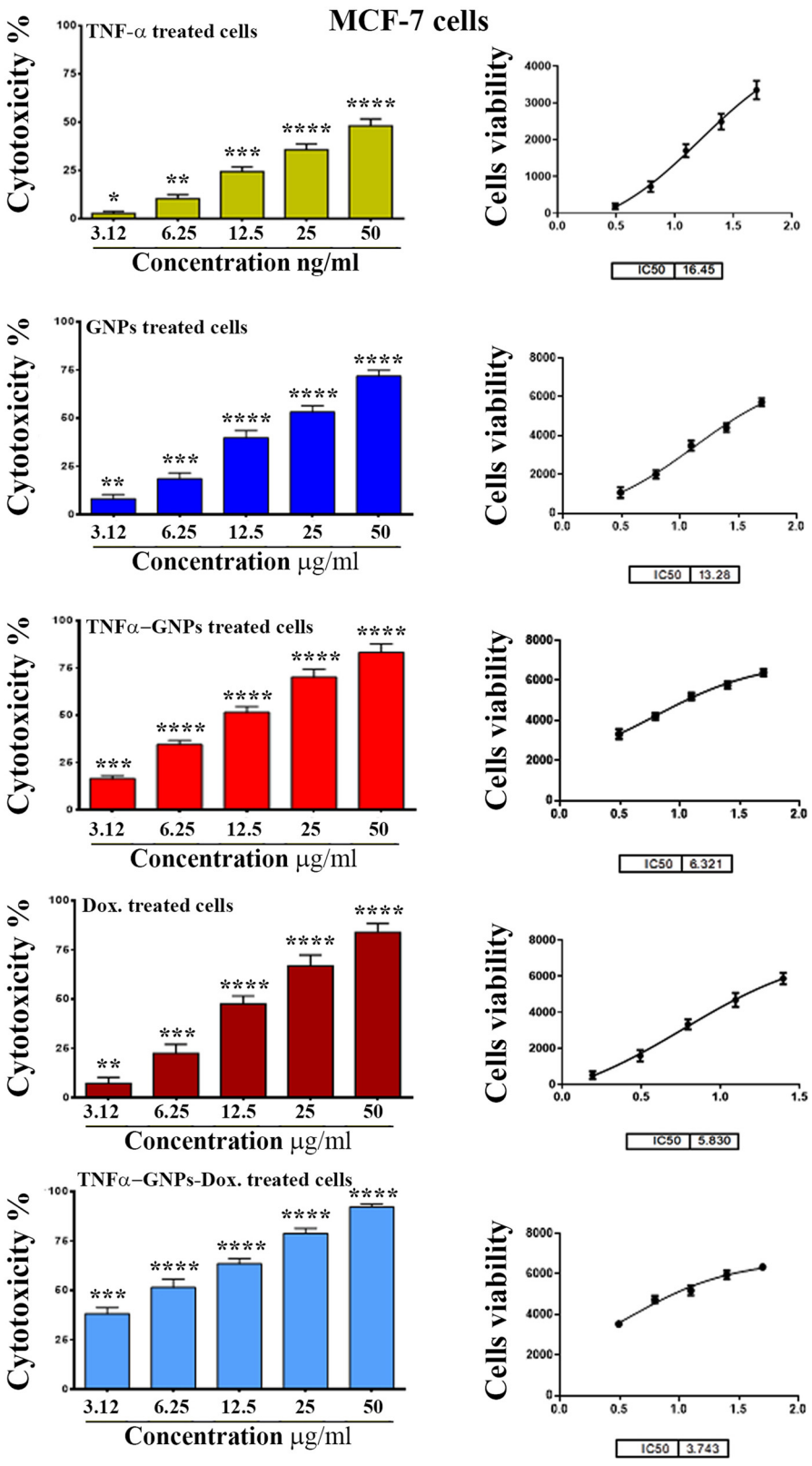

Figure 1: Anti-proliferative activity of GNPs loaded with TNF-α alone, doxorubicin alone, and both as a combined therapy in MCF-7 cells. Data are represented as mean ± SD of three independent experiments. Asterisks indicate statistically different from control untreated. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

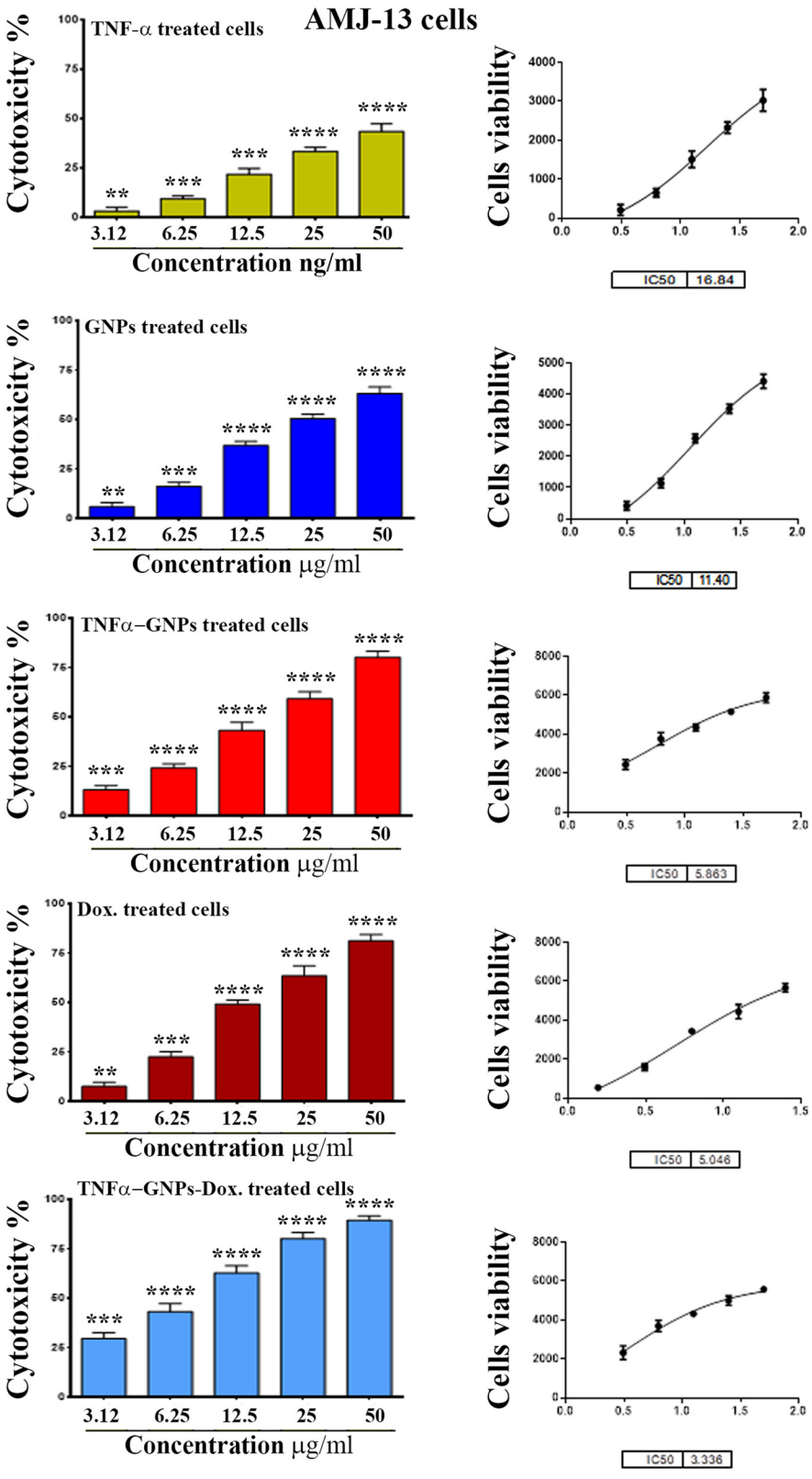

Figure 2: Anti-proliferative activity of GNPs loaded with TNF-α alone, doxorubicin alone, and both as a combined therapy in AMJ-13 cells. Data are represented as mean ± SD of three independent experiments. Asterisks indicate statistically different from control untreated. $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

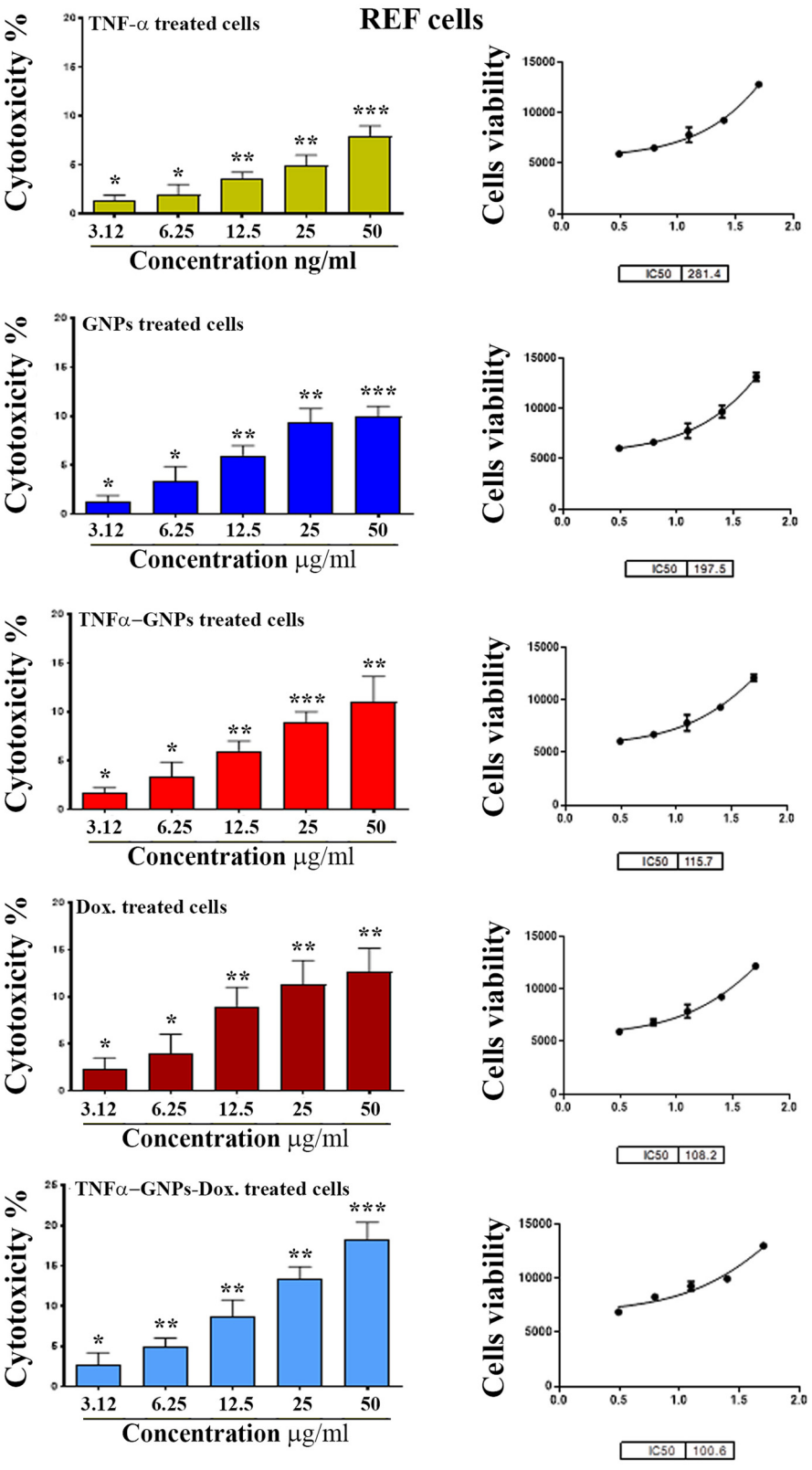

Figure 3: Anti-proliferative activity of GNPs loaded with TNF-α alone, doxorubicin alone, and both as a combined therapy in REF cells. Data are represented as mean ± SD of three independent experiments. Asterisks indicate statistically different from control untreated. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

tumor effects than resveratrol *in vitro* and *in vivo*. It is possible that this is due to the fact that GNPs carry more resveratrol into cells and are positioned in mitochondria. These findings suggest that Res-GNPs have anticancer effects that are much better than those of Res alone, both *in vitro* and *in vivo*, and that they may be useful for the clinical treatment of liver cancer [46]. PEG-gold nanoparticles (PEGAuNPs, 24 nm, 9.8 nM) have a very good cytotoxic effect on pancreatic cancer cells [47]. This is especially true when they are paired with chemotherapeutic medicines (doxorubicin or varlitinib). The proportion of cytotoxic activity and apoptosis of two human breast cancer cell lines (MCF-7 and MDA-MB-231) increased when the toxin from Naja naja venom (NN-32, IC_{50}: 5.0 g/mL) was coupled with GNPs (18 nm) [48].

The cytotoxic effect of GNPs loaded with TNF-α alone, doxorubicin alone, and both as a combined therapy using a colony-forming assay was confirmed. The results showed that GNPs loaded with TNF-α alone, doxorubicin alone, and both as combined therapy have high activity in suppressing colony-formation cells in comparison with control untreated MCF-7 and AMJ-13 cells, as shown in Figure 4. The decrease in the colony formation of breast cancer cells suggested that the cells that were exposed to continuous treatment were killed within 24–48 h, which suggested that GNPs loaded with TNF-α, doxorubicin alone, and both combined were taken up by breast cancer cells, which led to the induction of apoptotic. Furthermore, AO/EtBr stain was used to study the nuclear morphology of the breast cancer cells after treatment with GNPs loaded with TNF-α, doxorubicin alone, and both combined. After being stained with AO-EtBr, cells that had not undergone apoptosis were green in color, whereas apoptotic cells had an orange or red color. As shown in Figure 5, cells that were treated with GNPs loaded with TNF-α, doxorubicin alone, and both combined had many more apoptotic cells than the control untreated cells. When breast cancer cells were treated with GNPs loaded with TNF-α alone, doxorubicin alone, and both as a combined therapy, the results of the current study demonstrate that the viability of MCF-7 and AMJ-13 cells was significantly decreased. Bisht *et al.* demonstrated that a high dose of ZnO-Fe3O4 magnetic composite nanoparticle induces a cytotoxic effect in human breast cancer cell line (MDA-MB-231) but did not induce this effect in normal mouse fibroblast (NIH 3T3) [49].

3.2 Apoptosis proteomic profile

To examine the mechanism of breast cancer cell death induced by GNPs loaded with TNF-α alone, doxorubicin alone, and both as a combined therapy, an apoptosis protein array was used to study apoptosis protein expression in treated and untreated breast cancer MCF-7 and AMJ-13 cells, as shown in Figure 6. In this study, a Human Apoptosis protein array was used to measure the expression of some of the proteins involved in cell death and apoptosis after 24-h treatment with GNPs loaded with TNF-α alone, doxorubicin alone, and both as a combined therapy. Changes with upregulated proteins were also observed. Moreover, several such proteins, which include Bim, BAX, SMAC, Bad, cytochrome c, and HtrA-2, are essential components of the intrinsic apoptotic pathway; after 24 h

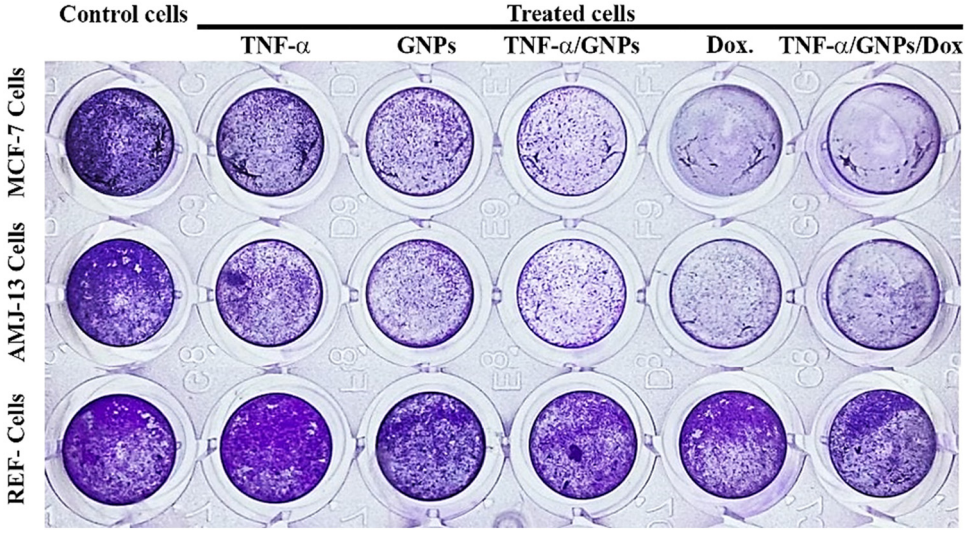

Figure 4: Effect of GNPs loaded with TNF-α alone, doxorubicin alone, and both as a combined therapy in colony forming of breast cancer cells.

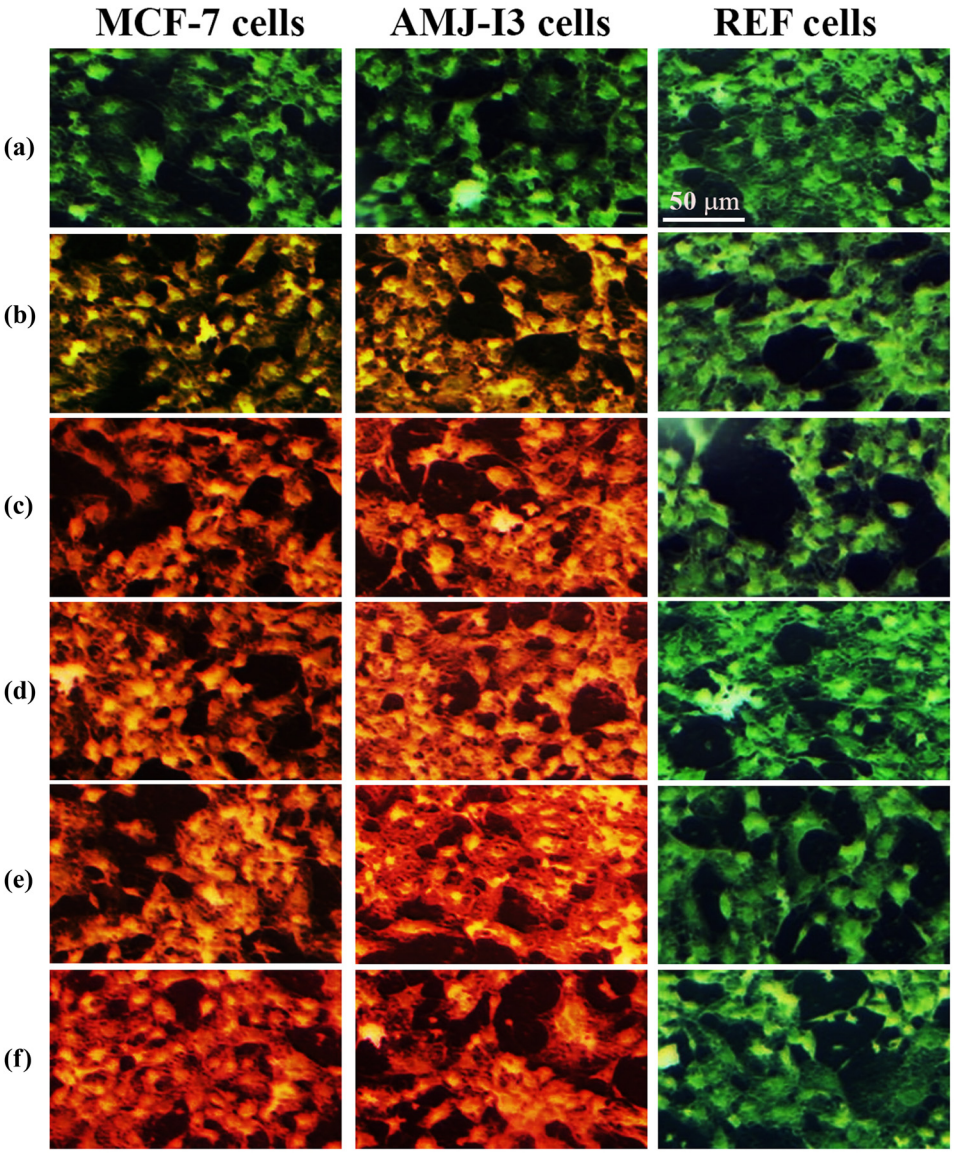

Figure 5: Detection of apoptosis using AO/EtBr in breast cancer cell lines. (a) Control untreated cells. (b) TNF-α-treated cells. (c) GNPs-treated cells. (d) GNPs–TNF-α-treated cells. (e) Doxorubicin-treated cells. (f) GNPs–TNF-α–doxorubicin-treated cells. Scale bar = 50 μm.

treated with GNPs loaded with TNF-α alone, doxorubicin alone, and both as a combined therapy, these proteins were significantly upregulated. BAD, Caspase-3, Caspase-7, and p53. Numerous studies have demonstrated that Bcl-2 proteins, including BAX, Bim, Bad, Bcl-2, and Bcl-w, are essential components of the mitochondrial pathway. These proteins transport cytochrome c from the mitochondria to the cytoplasm of the cell. This results in the generation of an apoptosome and the stimulation of caspase-9's downstream molecules, which facilitate the signaling of caspase-3 and caspase-7 [50]. The finding of the investigation on the expression of cellular proteins indicates that the apoptotic process is endogenous. In addition, research done in the past has shown that the tumor suppressor protein p53 is essential for causing apoptosis. Moreover, p53 may work with the mitochondrial pathway or stop the cell cycle by controlling the Bcl2 protein family or by causing the expression of p21 [51]. Furthermore, p53 is involved in the production of p27, which interacts with the Bax protein and makes apoptosis happen more quickly [52]. The results of this study showed that MCF-7, AMJ-13 cells treated with GNPs loaded with TNF-α alone, doxorubicin alone, and both as a combined therapy can induce apoptosis *via* the intrinsic, or *via* the mitochondrial pathway. The apoptosis process in breast cancer cell lines treated with GNPs loaded with TNF-α alone, doxorubicin alone, and both as a combined therapy was confirmed using

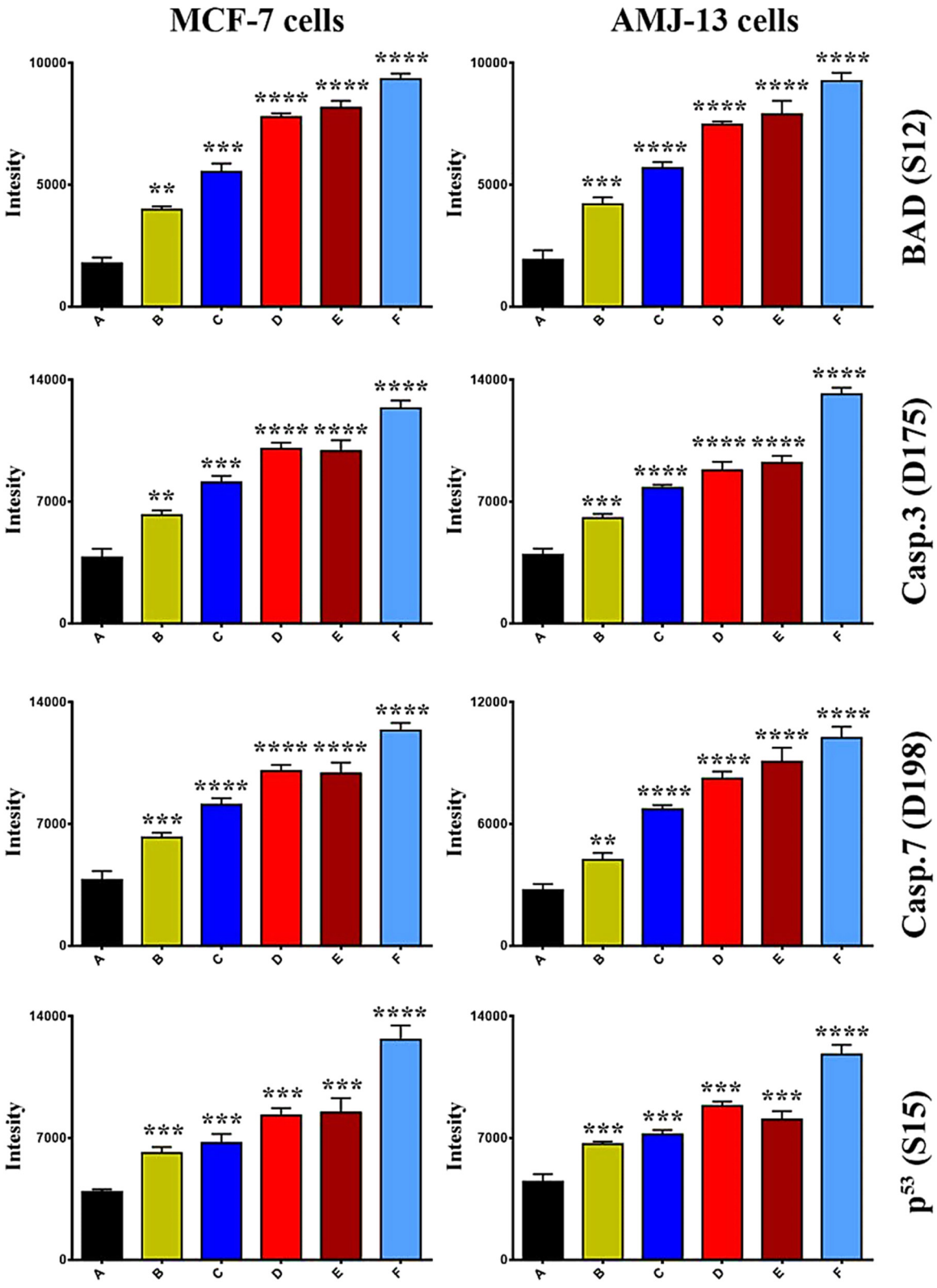

Figure 6: GNPs loaded with TNF-α and combined doxorubicin upregulated apoptosis pathways proteins. (A) Control untreated cells. (B) TNF-α-treated cells. (C) GNPs-treated cells. (D) GNPs–TNF-α-treated cells. (E) Doxorubicin-treated cells. (F) GNPs–TNF-α–doxorubicin-treated cells. Data are represented as mean ± SD. Asterisks indicate statistically different from control untreated. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

an immunofluorescent assay of effector caspases (caspase-3) expression. This was done because some caspases are not involved in the initiation of the apoptosis signal but rather are involved in signaling which leads to cytokines production during the inflammation process and other types of cell death. Figure 7 indicates that cleavage of caspase-3 was caused by exposure to GNPs loaded with TNF-α alone, doxorubicin alone, and both as a combined therapy. The outcomes suggest that GNPs loaded with TNF-α alone, doxorubicin alone, and both as a combined therapy were responsible for the induction of cell death in MCF-7 and AMJ-13 cells through the activation of a pathway involving caspase-dependent apoptotic signaling.

3.3 GNPs loaded with TNF-α and doxorubicin-induced mitochondrial dysfunction

The generation of ROS and their accumulation in mitochondria lead to a reduction in MMP, which triggers the mitochondrial apoptotic pathway [53]. JC-1 staining was performed on MCF-7 and AMJ-13 cells to assess the number of healthy and damaged mitochondria. The effect of GNPs loaded with TNF-α and doxorubicin was evaluated on both breast cancer cell lines. Flow cytometry was used to examine the results. For the purpose of determining whether or not mitochondrial damage has occurred, it is known that MMP ($\Delta\Psi m$) is produced by the proton pump of the electron transport chain, which is a component that is required for the production of ATP. For this reason, additional assessment for MMP was utilized using JC-1 staining. As shown in Figure 8, the promotion of JC-1 monomers noticeably increased depending on the type of treatment that was given to the breast cancer cell lines. It was demonstrated that when the cells were treated with combined therapy, the value of MMP significantly decreased. Our findings indicated that GNPs loaded with TNF-α alone, doxorubicin alone, and both as a combined therapy caused mitochondrial damage in human breast cancer cells, which led to the subsequent release of cytochrome c that, in turn, led to the activation of the caspase-9 and caspase-3 pathway.

3.4 GNPs loaded with TNF-α and doxorubicin induce autophagy in breast cancer cells

The expression of LC3, which is an important autophagy-related protein was investigated using a flow cytometry assay to determine whether or not autophagy is induced in GNPs loaded with TNF-α alone, doxorubicin alone, and both as a combined therapy for treating breast cancer cells. As shown in Figure 9, MCF-7 and AMJ-13 cells were exposed to GNPs loaded with TNF-α alone, doxorubicin alone, and both as a combined therapy, and there was an increase in

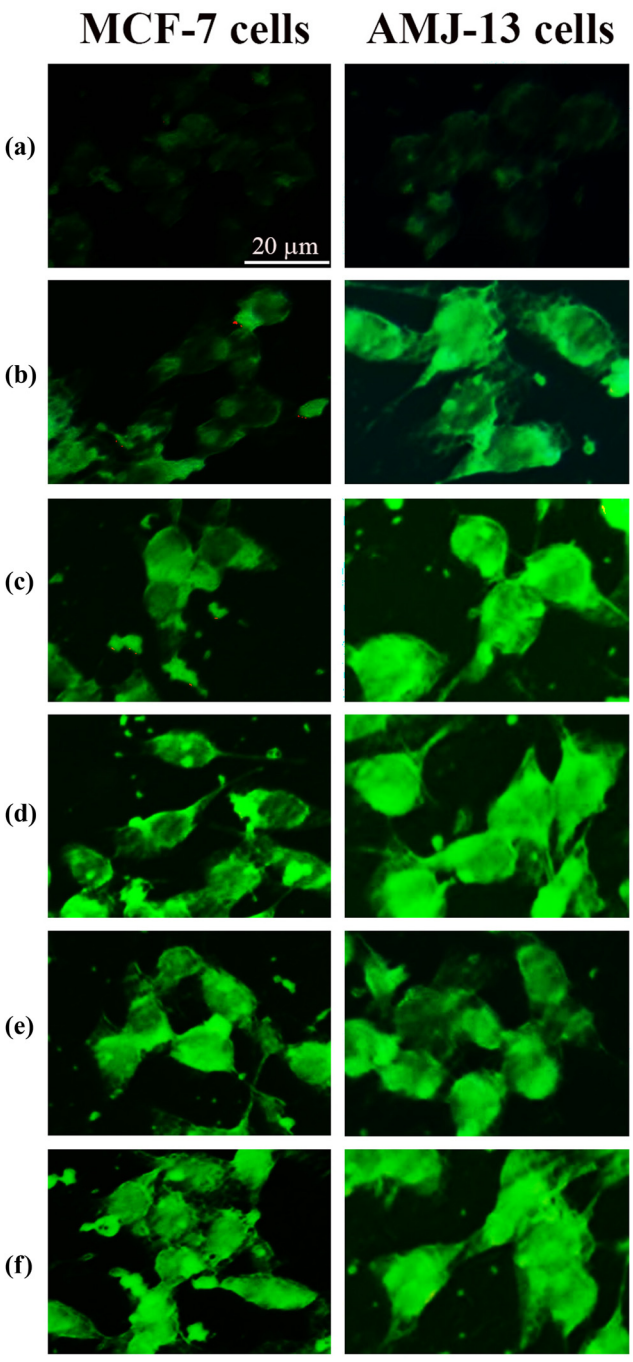

Figure 7: GNPs loaded with TNF-α and combined with doxorubicin induce cleaved-caspase-3 in breast cancer cells. (a) Control untreated cells. (b) TNF-α-treated cells. (c) GNPs-treated cells. (d) GNPs–TNF-α-treated cells. (e) Doxorubicin-treated cells. (f) GNPs–TNF-α–doxorubicin-treated cells.

the expression of the LC3 protein. The expression of LC3 was shown to be higher in GNPs loaded with TNF-α and doxorubicin as compared to control cells that had not been treated. Then, the ability of GNPs loaded with TNF-α and doxorubicin to induce autophagy in breast cancer cell lines was shown by the appearance of LC3 autophagosomes. To confirm the role of Beclin1 in the induction of autophagy in breast cancer cell lines after being treated with GNPs loaded with TNF-α and doxorubicin, Beclin1 was measured by using an immunofluorescent assay, as shown in Figure 10. In recent years, researchers have discovered that a wide range of nanomaterials can trigger autophagy. A previous study demonstrated that silver nanoparticles (AgNPs) have a significant therapeutic potential against a wide variety of cancer cells. This was accomplished by modulating the action of autophagy either as cytotoxic agents or as nanocarriers that, in conjunction with other treatments, deliver therapeutic molecules [54]. AgNPs were shown to be possible sources of oxidative stress, which, when exposed to NIH3T3 cells, results in the generation ROS and, ultimately, the induction of autophagy [55]. However, despite the fact that this nanomaterial was subjected to extensive research for its powerful lethal effect in a wide range of tested cancer cell lines [56], there is a possibility that it activates an autophagy process in human keratinocyte cells derived from HaCaT [57]. It was demonstrated that RAW264.7 cells originating from mouse peritoneal macrophages can be stimulated to undergo autophagy when exposed to Fe3O4-NPs. Following treatment with Fe_3O_4-NPs, there was an increase in autophagy markers and levels of ROS [58]. In a study [59], the authors discussed the potential effect and underlying mechanism of nanomaterials on the polarization of tumor-associated macrophages (TAMs). They used PEG-AuNPs as a model nanomaterial due to their biocompatibility as well as colloidal stability. According to their findings, PEG-AuNPs elicited antitumor immunotherapy by preventing the polarization of TAMs toward the M2 state through autophagy interference. PEG-AuNPs have the potential to promote autophagic flux suppression in TAMs. This is due to the fact that PEG-AuNPs stimulate lysosome alkalization and membrane permeabilization in TAMs. In addition, after autophagy was activated, TAMs polarized toward the M2 phenotype; however, inhibiting autophagic flux could reduce the M2 polarization of TAMs.

3.5 Apoptosis and autophagy induced *via* AKT and JNK signaling pathways

Mitogen-activated protein kinases (MAPKs) are key regulatory mechanisms that play a significant part in the

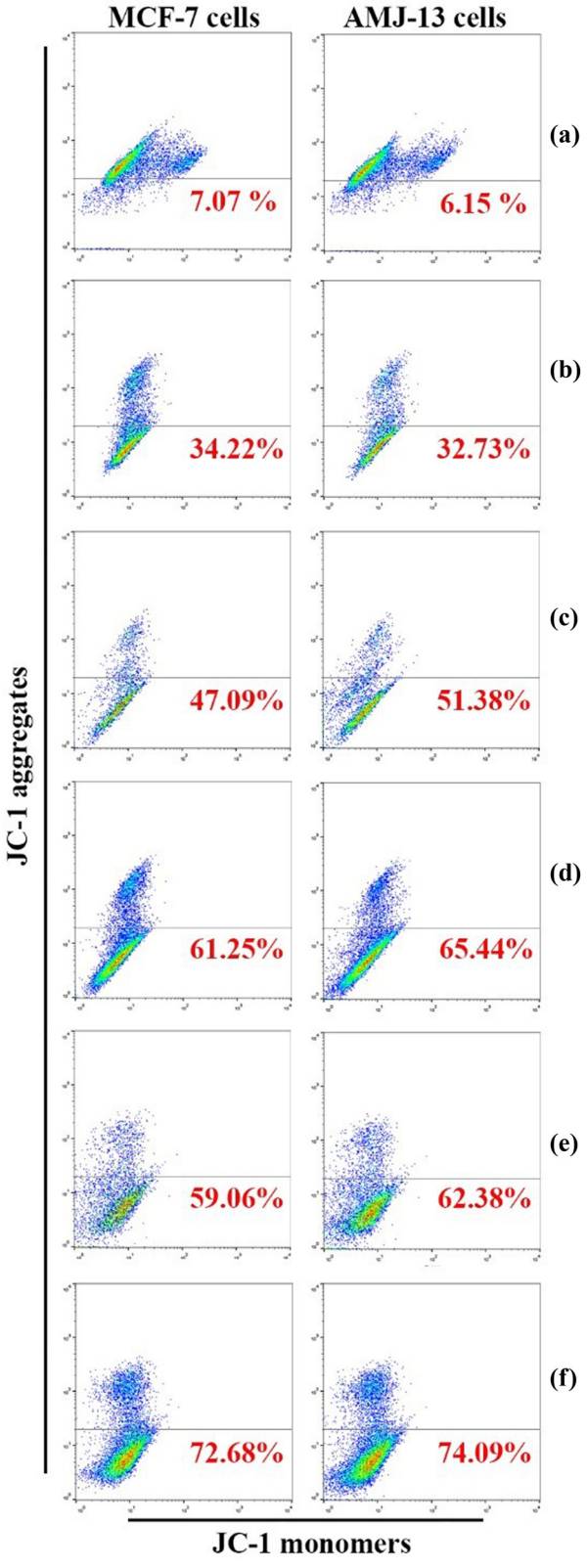

Figure 8: GNPs loaded with TNF-α and combined with doxorubicin reduce MMP. (a) Control untreated cells. (b) TNF-α-treated cells. (c) GNPs-treated cells. (d) GNPs–TNF-α-treated cells. (e) Doxorubicin-treated cells. (f) GNPs–TNF-α–doxorubicin-treated cells.

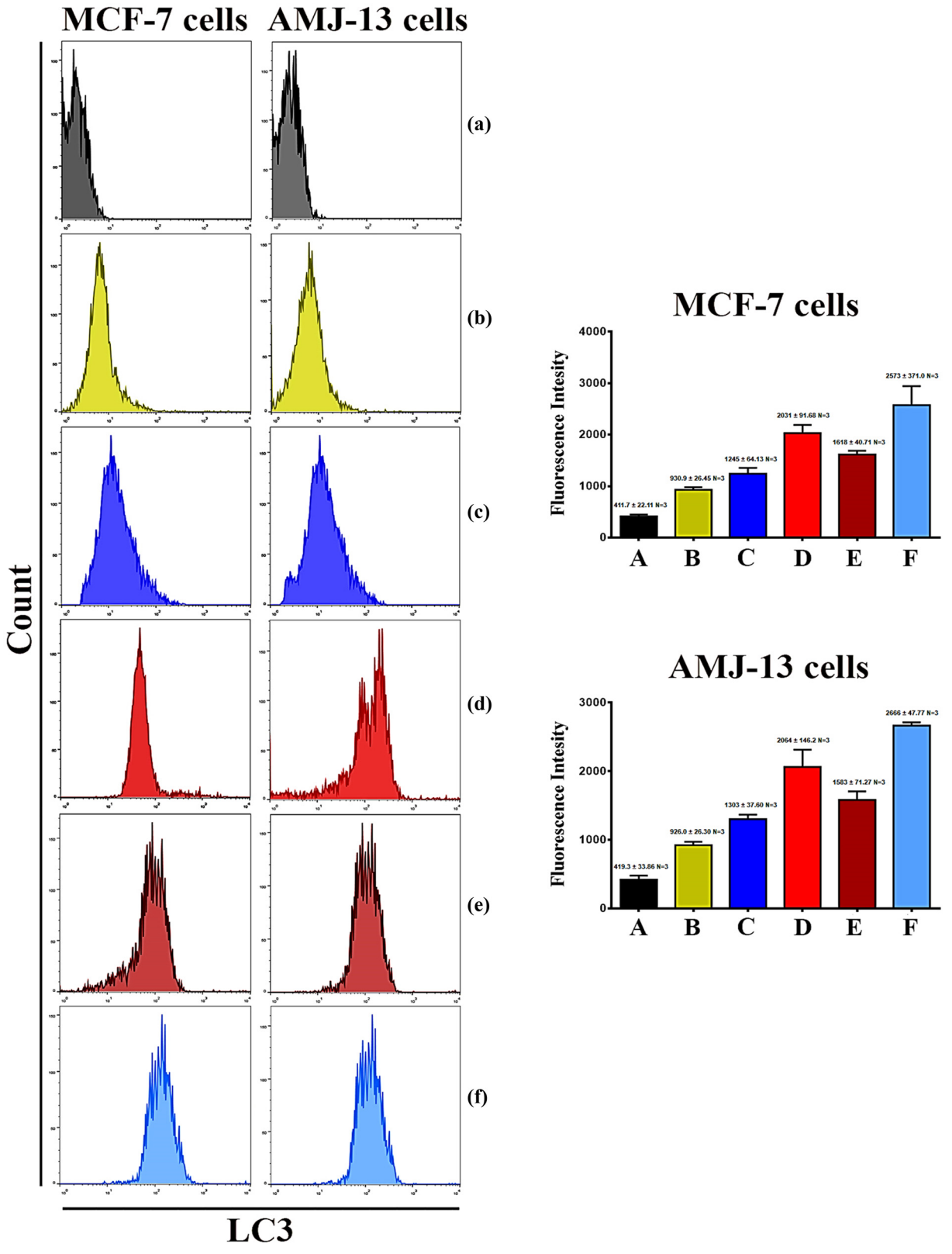

Figure 9: GNPs loaded with TNF-α and combined with doxorubicin induce autophagy in breast cancer cell lines. (a) Control untreated cells. (b) TNF-α-treated cells. (c) GNPs-treated cells. (d) GNPs–TNF-α-treated cells. (e) Doxorubicin-treated cells. (f) GNPs–TNF-α–doxorubicin-treated cells. Data are represented as mean ± SD of three independent experiments.

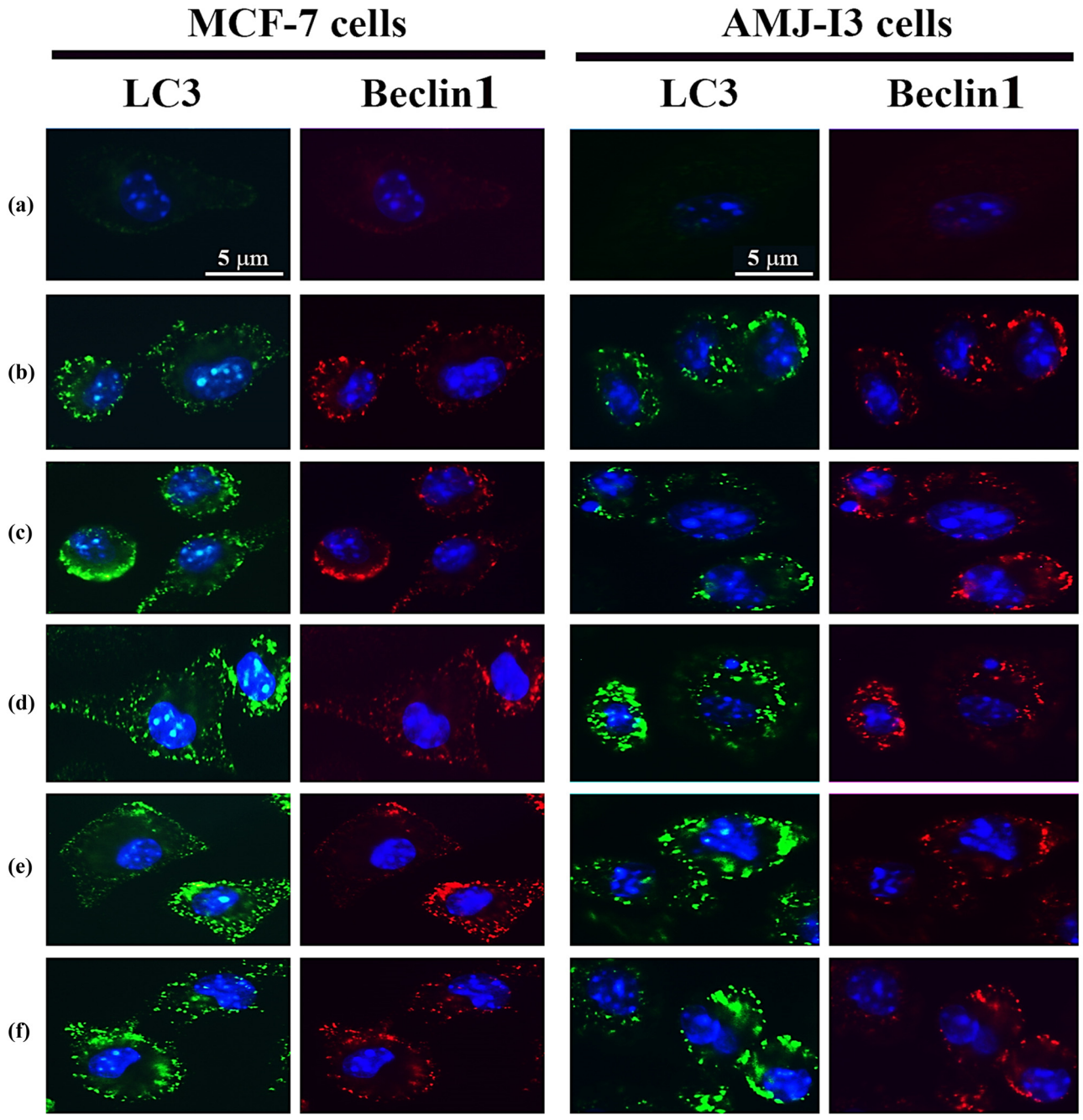

Figure 10: Autophagy markers LC3 and Beclin1in breast cancer cell lines. (a) Control untreated cells. (b) TNF-α-treated cells. (c) GNPs-treated cells. (d) GNPs–TNF-α-treated cells. (e) Doxorubicin-treated cells. (f) GNPs–TNF-α–doxorubicin-treated cells.

biological translation of cell autophagy and apoptosis [60]. These processes are found in eukaryotic cells. MAPK pathways are a type of serine/threonine protein kinase that are commonly present in prokaryotic and mammalian cells. These pathways are continually involved in the processes of gene expression, cell division, differentiation, apoptosis, autophagy, and even cancer cell migration, invasion, and other forms of carcinogenesis [61]. Hence, using an immunofluorescent assay, the levels of protein expression of MAPKs, such as p-JNK1/2, and p-AKT, in breast cancer cell lines that had been treated with GNPs loaded with TNF-α alone, doxorubicin alone, and both as a combined therapy was measured. According to the findings, p-AKT was found to be decreased in both breast cancer cell lines, while p-JNK was found to be increased, as shown in Figure 11. A previously published study demonstrated that GNPs induced

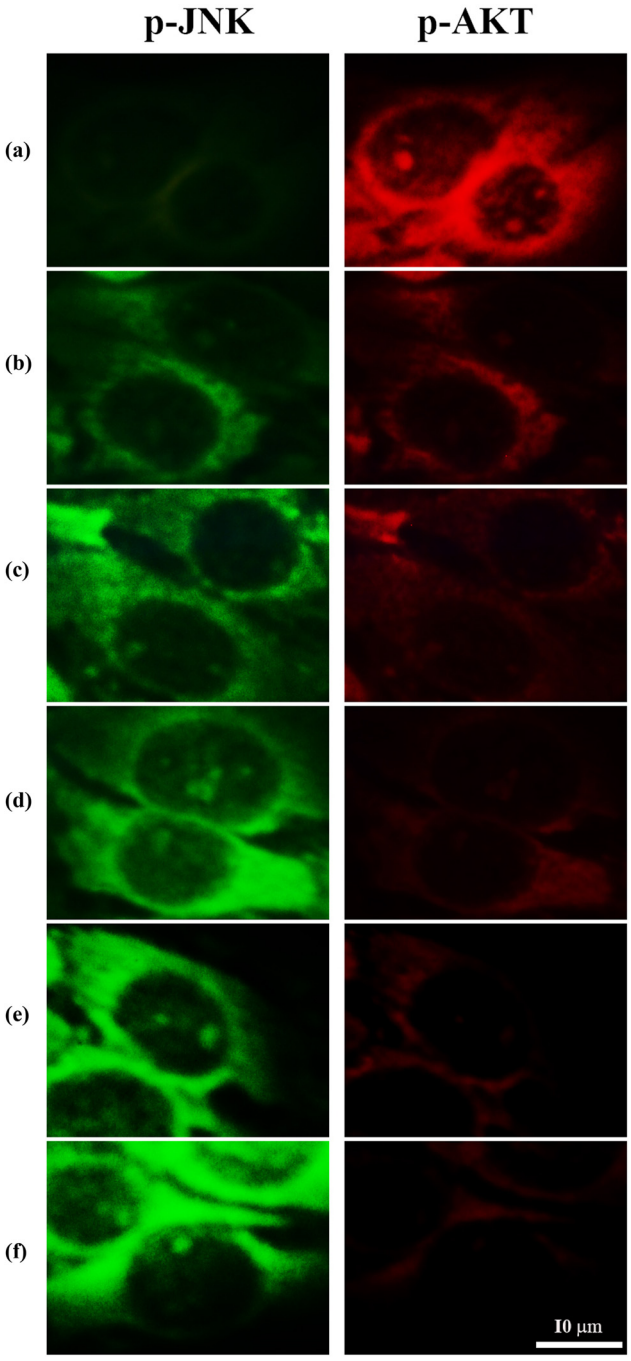

Figure 11: GNPs loaded with TNF-α and combined with doxorubicin induce apoptosis and autophagy by controlling AKT and JNK MAPK Pathways. (a) Control untreated cells. (b) TNF-α-treated cells. (c) GNPs-treated cells. (d) GNPs–TNF-α-treated cells. (e) Doxorubicin-treated cells. (F) GNPs–TNF-α–doxorubicin-treated cells. Scale = bare 10 µm.

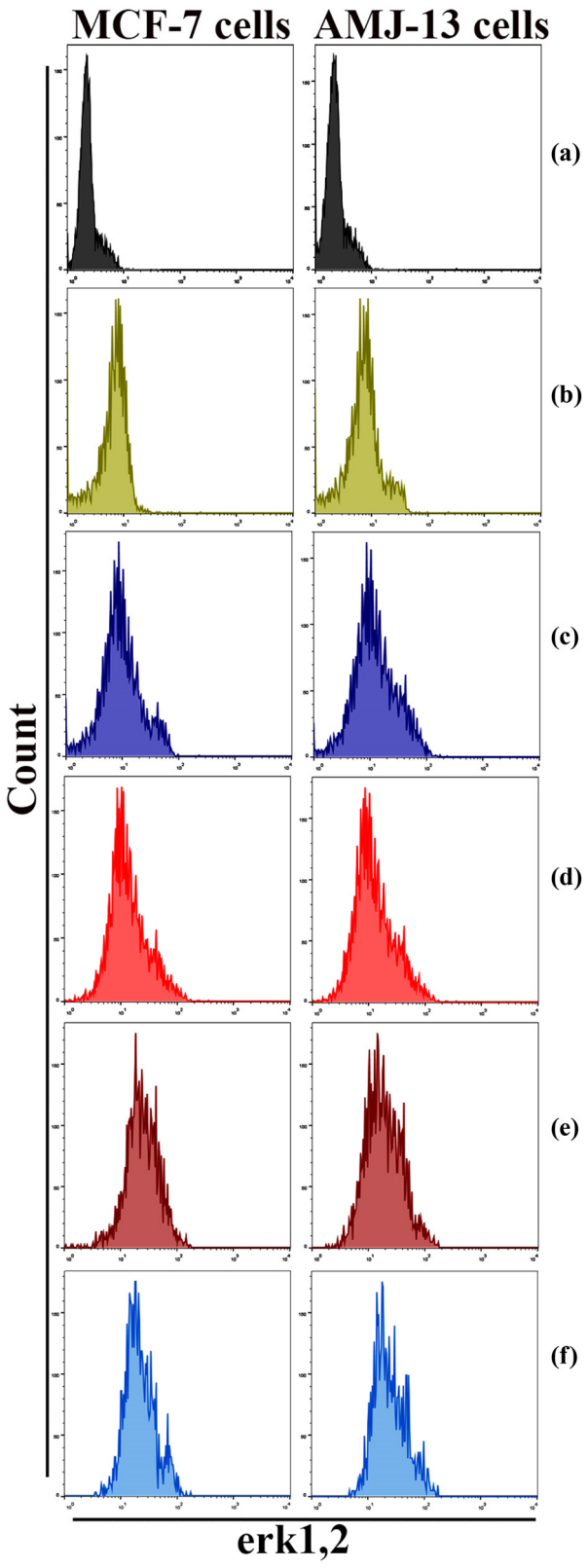

Figure 12: GNPs loaded with TNF-α and combined with doxorubicin induce autophagy *via* erk 1, 2 pathways. (a) Control untreated cells. (b) TNF-α-treated cells. (c) GNPs-treated cells. (d) GNPs–TNF-α-treated cells. (e) Doxorubicin-treated cells. (f) GNPs–TNF-α–doxorubicin-treated cells.

apoptosis and autophagy in ovarian cancer cells (SKOV-3); these findings refer to NPs that can increase ROS generation and activate JNK and p38 [62]. Based on our findings, it appears that the activation of AKT and JNK1/2 may play a role in the regulation of GNPs loaded with TNF-α and

doxorubicin-induced autophagy and apoptosis. The previous study demonstrated that the ROS-dependent ERK activation could induce cell apoptosis and cell cycle arrest [63]. ERK signaling pathway play an important role in autophagy process [64]. In the current study, we shown that the ERK signaling and p-mTOR protein expression was suggestively improved *via* exposed breast cancer cell lines to GNPs, TNF-α alone, doxorubicin alone, and as a combined therapy as indicated in Figure 12.

4 Conclusions

The present study aimed to estimate the anti-proliferative activity of GNPs loaded with TNF-α combining doxorubicin against breast cancer cell lines, as well as promoted the induction of apoptosis proteins in MCF-7 and AMJ-13 cells. GNPs loaded with TNF-α combined doxorubicin had synergistic effects on both cell lines through caspases induction involvement and their ability to induce a wide range of apoptotic proteins. These apoptosis proteins included Bad, caspase-3, caspase-7, and p53. GNPs loaded with TNF-α combining doxorubicin upregulated LC3-II and Beclin1 proteins expression in both MCF-7 and AMJ-13 cell lines, demonstrating that the GNPs loaded with TNF-α combining doxorubicin can induce autophagy. The outcomes demonstrated that GNPs loaded with TNF-α and doxorubicin had induced MAPK signaling pathway *via* reduced p-AKT, while simultaneously increasing p-JNK1/2 activities. The findings of the present study suggest that GNPs loaded with TNF-α combining doxorubicin induce both autophagy and apoptosis in breast cancer cell lines, and it is predictable to offer a therapeutic option as a future therapeutic approach against cancer cell proliferation.

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