

Apoptosis in human liver carcinoma caused by gold nanoparticles in combination with carvedilol is mediated via modulation of MAPK/Akt/mTOR pathway and EGFR/FAAD proteins

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Abstract. In cancers, apoptosis signaling pathways and cell survival and growth pathways responsible for resistance to conventional treatments, such as Pi3K/Akt/mTOR and mitogen-activated protein kinase (MAPK) become dysregulated. Recently, alternative treatments to promote tumor cell death have become important. The present study reports on the antitumor and cytoprotective action of gold nanoparticles (GNPs) and carvedilol in combination and in isolated application. Apoptosis was analyzed by FITC/propidium iodide staining flow cytometry; caspase-3, caspase-8, Bcl-2 and MAPK/ERK activity by immunofluorescence microscopy; gene expression of proteins related to cell death as Akt, mTOR, EGFR, MDR1, survivin, FADD and Apaf, by the

real-time PCR; and western blot analysis for MAPK/ERK, Akt and mTOR. Oxidative stress evaluation was performed by reduced glutathione (GSH) and malondialdehyde (MDA) levels. Intracellular GNPs targets were identified by transmission electron microscopy. After exposure to a combination of GNPs (6.25 μ g/ml) and carvedilol (3 μ M), death as promoted by apoptosis was detected using flow cytometry, for expression of pro-apoptotic proteins FADD, caspase-3, caspase-8 and sub-regulation of anti-apoptotic MAPK/ERK, Akt, mTOR, EGFR and MDR1 resistance. Non-tumor cell cytoprotection with GSH elevation and MDA reduction levels was detected. GNPs were identified within the cell near to the nucleus when combined with carvedilol. The combination of GNP and carvedilol promoted downregulation of anti-apoptotic and drug resistance genes, over-regulation of pro-apoptotic proteins in tumor cells, as well as cytoprotection of non-tumor cells with reduction of apoptosis and oxidative stress.

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Abbreviations: GNPs, gold nanoparticles; Carv, carvedilol; EGFR, epidermal growth factor receptor; Erk, extracellular signal-regulated kinases; FADD, fas-associated protein with death domain; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; MDA, malondialdehyde; MAPK, mitogen activated protein kinases; MDR1, multidrug resistance genes-1; mTOR, mechanistic target of rapamycin; Pi3K, phosphatidylinositol 3-kinases

Key words: apoptosis, cancer, gold nanoparticles, carvedilol, combination

Introduction

Cancer, a serious public health problem worldwide, is responsible for countless deaths each year and is currently considered the second leading cause of death on the planet (1-3). In addition, various malignant tumor types do not have effective treatment (4-8), due to the ability of tumor cells to evade death, by presenting changes in apoptosis pathway protein levels (9-11). Changes in other cellular pathway proteins such as Pi3k/Akt/mTOR and MAPK/ERK, which are highly dysregulated in malignant tumors, also corroborate the ability of malignant cells to evade apoptosis death, contributing to chemotherapy resistance (12-14). The expression of multi-drug resistance genes, such as MDR1, has been implicated

as the main cause of chemoresistance (15,16). Furthermore, conventional chemotherapeutic treatments are known for their side-effects on non-tumor cells, such as the strong oxidative stress (17-19).

Moreover, there is a need for alternative treatments that promote apoptosis in tumor cells and yet do not negatively affect normal cells. In this regard, GNPs have been highlighted in the literature as promising agents with large pooling surfaces for various drugs (20), which concentrate on tumor tissues (21,22), are resistant to corrosion and present low toxicity to the biological system (23-25). In addition, various studies reported an effective antitumor action with non toxicity to normal cells (26-29). The antihypertensive carvedilol is often used as a non-selective inhibitor of adrenergic receptors (30), is known for its cardiovascular and antioxidant benefits (31-34) and has recently presented good antitumor activity, such as growth inhibition of neuroblastoma cell lines (35) and rat glioma cell line (36), suppressing migration and invasion of malignant breast cells (37), preventing carcinogenesis in rat epidermal lineages (38) and promoting apoptosis in tumoral hepatic and oral cell lineages (39,40). The discovery of new substances and combinations is fundamental to the process of establishing new treatments against cancer (41-43). Combining substances has proven to be extremely effective due to the lower doses used, the decreased adverse effects, and the possibility of acting on different signaling pathways (44-50). In addition, the discovery of substances that promote inhibition of dysregulated pathways such as Pi3k/Akt/mTOR and MAPK/Erk would positively modulate apoptosis in tumor cells (12,51,52). In the present study, we investigated the effects of combined carvedilol and GNPs action on both tumor and non-tumor cells.

Materials and methods

Reagents. The reagents were purchased as indicated: Dulbecco's modified Eagle's medium (DMEM; Life Technologies, Grand Island, NY, USA); 10% (v/v) heat-inactivated fetal bovine serum (FBS; Cultilab Materiais para Cultura de Células Ltda, Campinas, Brazil); trypsin/ EDTA (ethylenediaminetetraacetic acid) (Gibco-BRL, Life Technologies, Grand Island, NY, USA); cisplatin (citoplax, 50 mg; Bergamo, Taboão da Serra, Brazil); gold nanoparticles GNPs (Institute of Chemical, UFRN, Natal, Brazil); carvedilol (Farmafórmula, Natal, Brazil); gold (III) chloride (30% wt. in HCl), sodium hydroxide, glycerol, and polyvinylpyrrolidone (PVP, molecular weight, 10,000 Da) were products from Sigma-Aldrich. The synthesis and characterization of GNPs was the described by de Araújo *et al* (53). GNPs, carvedilol and cisplatin solutions were filtered using a 0.22-mm minipore membrane.

Cell culture. The human cell lines hepatocellular carcinoma (HepG2) and human non-cancerous renal cell line (HEK-293) were purchased from the Culture Collection of the Federal University of Rio de Janeiro (RJC Collection, Rio de Janeiro, Brazil). HepG2 and Hek-293 cells were maintained in DMEM supplemented with 10% (v/v) heat-inactivated FBS.

Cell viability. In order to determine GNP and carvedilol doses to promote and maintain low inhibition in cancer cells, cell viability was determined by trypan blue exclusion assay. The viability was

determined at 24 and 48 h for HepG2 (1×10^5 cells) at different concentrations of GNPs (1-50 $\mu\text{g/ml}$, aqueous suspension) and carvedilol [1.5-300 μM , dissolved in dimethyl sulfoxide (DMSO) 1%]. The cells were placed into 6-well plates. Briefly, cell aliquots were mixed with the same volume 0.5% (w/v) trypan blue and incubated at room temperature for 5 min. The number of viable cells was calculated using a hemocytometer.

Annexin V and propidium iodide staining. The apoptotic assay was conducted according to Araújo Jr *et al* (54). HepG2 and HEK-293 were plated in 6-well plates (2×10^5 cells/well) with 2 ml medium/well. After 24 h, concentrations of GNPs (3 and 6.25 $\mu\text{g/ml}$), cisplatin (15 $\mu\text{g/ml}$) and carvedilol (1.5 and 3 μM) were added (24 and 48 h), respectively. In parallel, control cells were maintained in culture medium without GNPs, carvedilol or cisplatin. For observation of combined action, the cells were treated with GNPs (3 and 6.25 $\mu\text{g/ml}$) and at 24 h treated with carvedilol (1.5 and 3 μM). After another 24 h, they were analyzed. The cells were then assayed using the Annexin V-FITC/PI apoptosis detection kit I (BD Biosciences, San Diego, CA, USA). Annexin V-FITC and propidium iodide (PI) were added to the cellular suspension according to the manufacturer's instructions. A total of 1×10^6 cells from each sample was then analyzed by FACSCalibur cytometer (BD Biosciences, Franklin Lakes, NJ, USA), and FlowJo software (BD Biosciences). Annexin V-FITC-positive/PI-negative cells were identified as cells in the early stages of apoptosis, while Annexin V-FITC-positive/PI-positive cells were identified as cells in the late stages of apoptosis, or as cells undergoing necrosis.

Glutathione (GSH) levels. Antioxidant GSH levels in cell lines were measured [adapted from Rahman *et al* (55) and Costa *et al* (56)]. HepG2 and Hek-293 were plated in 6-well plates (2×10^5 cells/well) with 2 ml medium/well. After 24 h, GNPs (6.25 $\mu\text{g/ml}$) was added and after 24 h, 3 μM carvedilol. A homogenate of cells (100 μl of cell in 500 μl EDTA 0.02 M) were added to 320 μl of distilled water and 80 μl of 50% trichloroacetic acid (TCA). Samples were centrifuged at 3,000 rpm for 15 min at 4°C. The supernatant (100 μl) was added to 200 μl of 0.4 M Tris buffer at pH 8.9 and 20 μl of 0.01 M 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB). The absorbance of each sample was measured at 420 nm, in a spectrophotometric/microplate reader Polaris and the results were reported as units of GSH per milligram.

Malondialdehyde levels. Malondialdehyde (MDA) is an end product of lipid peroxidation. To quantify the increase in free radicals in non-cancer (HEK-293) and cancer (HepG2) cells, MDA content was measured via the assay described by Esterbauer and Cheeseman (57). Cell samples were suspended in buffer; Tris HCl 1:5 (w/v), and minced with scissors for 15 sec on an ice-cold plate. The resulting suspension was homogenized for 2 min with an automatic Potter homogenizer and centrifuged at 11,000 rpm at 4°C for 10 min. The supernatants were assayed in order to determine the MDA content. The absorbance of each sample was measured at 586 nm. The results are expressed as nanomoles of MDA per cell.

Immunofluorescence, Bcl-2, MAPK/ERK, caspase-3 and caspase-8 activity. HepG2 cells were plated on glass

coverslips in 24-well plates (5×10^4 cells/well). After 24 h, they were treated with the GNPs (6.25 $\mu\text{g/ml}$), cisplatin (15 $\mu\text{g/ml}$) and carvedilol (3 μM) for 48 h. For combined action, we used GNPs (6.25 $\mu\text{g/ml}$) + carvedilol (3 μM). The cells were then washed, and fixed with paraformaldehyde, permeabilized by Triton-X, and incubated with anti-Bcl-2 mouse polyclonal antibody, rabbit polyclonal anti-caspase-3 antibody (Abcam, San Francisco, CA, USA), rabbit anti-caspase-8 monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and monoclonal mouse antibody anti-MAPK/ERK (Invitrogen, Carlsbad, CA, USA) diluted 1:500 in phosphate-buffered saline (PBS) containing bovine serum albumin (BSA; 5%; Life Technologies do Brasil Ltda, São Paulo, Brazil) for 1 h at RT in a humid atmosphere. The primary antibody was detected with Alexa Fluor 488 goat anti-rabbit or anti-mouse secondary antibody (Abcam), and 4',6-diamidino-2-phenylindole (Life Technologies do Brasil Ltda) was used for nuclear staining. The immunostained coverslips were examined under Axio Observer z.1, inverted fluorescence and brightfield.

Fluorescent images were obtained on a Carl Zeiss Laser Scanning Microscope (LSM 710, 20X objectives; Carl Zeiss, Oberkochen, Germany). Negative controls and treated groups were included in each batch of samples. Cell reactivity in all groups (negative, GNPs, carvedilol, GNPs + carvedilol and cisplatin) was assessed by computerized densitometric analysis of the captured digital images with the aforementioned immunofluorescence microscope. Average densitometric values were calculated in ImageJ software (<http://rsb.info.nih.gov/ij/>). Contrast index measurements were obtained from the formula [(selected area \times 100)/total area] after removal of background in regions of interest (three samples per group).

Real-time PCR. HepG2 cells were plated in 6-well plates (2×10^5 cells/well) with 2 ml medium/well. After 24 h, concentrations of gold nanoparticles (6.25 $\mu\text{g/ml}$), cisplatin (15 $\mu\text{g/ml}$) and carvedilol (3 μM) were added for 48 h. For combined action, GNPs (6.25 $\mu\text{g/ml}$) + (3 μM) carvedilol was used. The cells were collected with cell scrapers and total RNA was isolated from cells using TRIzol reagent. The total RNA was extracted from cell samples using RNeasy Mini kit (Qiagen, Tokyo, Japan) from QIAcube following the manufacturer's guidelines. The total RNA extracted underwent reverse transcriptase activity using the High capacity RNA-to-cDNA kit (Applied Biosystems, Ltd., Tokyo, Japan). Real-time quantitative PCR analyses of EGFR, Akt, mTOR, survivin, MDR-1, FADD, Apaf-1 and GAPDH mRNAs were performed with SYBR-Green Mix in the Applied Biosystems® 7500 FAST system (Applied Biosystems, Foster City, CA, USA), according to a standard protocol with the following primers: *GAPDH* (forward, 5'-AAC TTT GGC ATC GTG GAA GG-3' and reverse, 5'-GTG GAT GCA GGG ATG ATG TTC-3', annealing primer temperature, 60°C); *EGFR* (forward, 5'-TGA TAG ACG CAG ATA GTC GCC-3' and reverse, 5'-TCA GGG CAC GGT AGA AGT TG-3', annealing primer temperature, 56.6°C); *Akt* (forward, 5'-ACG GCA TGG ACT TTA CCA AG-3' and reverse, 5'-GCG GGT GAA AGA CAG GAA TA-3', annealing primer temperature, 55°C); *mTOR* (forward, 5'-TTG AGG TTG CTA TGA CCA GAG AGA A-3' and reverse, 5'-TTA CCA GAA AGG ACA CCA GCC AAT G-3', annealing primer temperature, 58.3°C); *survivin* (forward, 5'-TAC AGC TTC

GCT GGA AAC CT-3' and reverse, 5'-AGC CCG GAT GAT ACA AAC AG-3', annealing primer temperature, 55.6°C); *MDR1* (forward, 5'-GTG TGG TGA GTC AGG AAC CTG TAT-3' and reverse, 5'-TCT CAA TCT CAT CCA TGG TGA CA-3', annealing primer temperature, 57°C); *FADD* (forward, 5'-TCT CCA ATC TTT CCC CAC AT-3' and reverse, 5'-GAG CTG CTC GCC TCC CT-3', annealing primer temperature, 58.7°C); and *Apaf-1* (forward, 5'-CCT CTC ATT TGC TGA TGT CG-3' and reverse, 5'-TCA CTG CAG ATT TTC ACC AGA-3', annealing primer temperature, 56.9°C). The experiments were performed in triplicate. The standard PCR conditions were as follow: 50°C for 2 min and 95°C for 10 min, followed by 40 of 30-sec cycles at 94°C, a variable annealing primer temperature for 30 sec and at 72°C for 1 min. Mean Ct values were used to calculate the relative expression levels of the target genes for the experimental groups as relative to those in the negative control group; expression data were normalized relative to the housekeeping gene *GAPDH* using the $2^{-\Delta\Delta C_t}$ formula.

Western blot analysis. Cells (HepG2) were lysed in buffer [Tris-HCl 50 mM, NaCl 150 mM, Triton X-100 1%, EDTA 1 mM, sodium pyrophosphate 20 mM, pH 7.4 containing a protease inhibitors cocktail (Roche), NaF (10 mM), DTT (1 mM), PMSF (0.1 mM) and sodium vanadate (1 mM)] on ice. To confirm equal loadings, total protein concentration was determined using the Bradford method (Bio-Rad Laboratories, Hercules, CA, USA). Proteins were resolved using SDS-PAGE and then transferred to a polyvinylidene difluoride (PVDF) membrane. Non-specific binding sites on the membrane were blocked using 5% non-fat skimmed milk and incubated with the primary antibody anti-Akt (1:500; Abcam), anti-mTOR (1:500; Abcam), and MAPK/ERK (1:200; Abcam), overnight at 4°C, followed by incubation with the appropriate secondary antibodies: Akt α -rat peroxidase 1:1,000; mTOR-rabbit peroxidase 1:2,000 and MAPK/ERK α -rat peroxidase 1:1,000. Proteins were detected using the ECL Plus kit (Perkin-Elmer, San Jose, CA, USA).

Transmission electronic microscopy (TEM). Cells (HepG2) at a density of 3×10^5 were plated into (GNPs sensitized and treated) 6-well plates, and after 48 h were collected with trypsin, centrifuged at 1,500 rpm for 5 min, and washed with PBS. The cell pellet was fixed with 2.5% glutaraldehyde + paraformaldehyde 4% + sodium cacodylate buffer (0.1 M) for 4 h at 4-8°C. Afterwards, the samples were washed in 0.05 M sodium cacodylate (3x30 min), post-fixed in 1% OsO_4 + 1% potassium ferrocyanide (2 h), washed again (3x) with 0.05 M sodium cacodylate (3x30 min), and then serially dehydrated in ethanol 50, 70, 90 and 100, for 30 min each. Polymerization of the resin was performed at 60°C for 48 h. Finally, ultramicrotomy was performed followed by staining (uranyl acetate 1% + 1% lead citrate 1 h), and electron microscope visualization (Tescan transmission, Vega 3 model).

Statistical analysis. All experiments were performed in triplicate, and the significant differences between the groups were calculated using the analysis of variance and the Bonferroni's test, as indicated. A $P < 0.05$ was considered statistically significant.

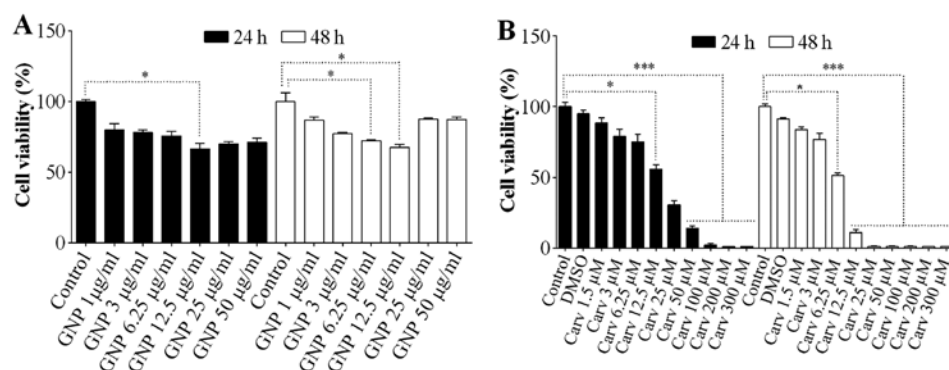


Figure 1. Cell viability to determine the cell growth. (A) Effect of different doses of GNPs on hepatic tumor cells (HepG2) at 24 and 48 h. The 12.5 $\mu\text{g/ml}$ dose caused greater cellular growth inhibition at both times. (B) Effect of different doses of carvedilol on hepatic tumor cells (HepG2) at 24 and 48 h. Doses $>6.25 \mu\text{M}$ promoted strong inhibition of cell growth. * $P<0.05$; *** $P<0.001$.

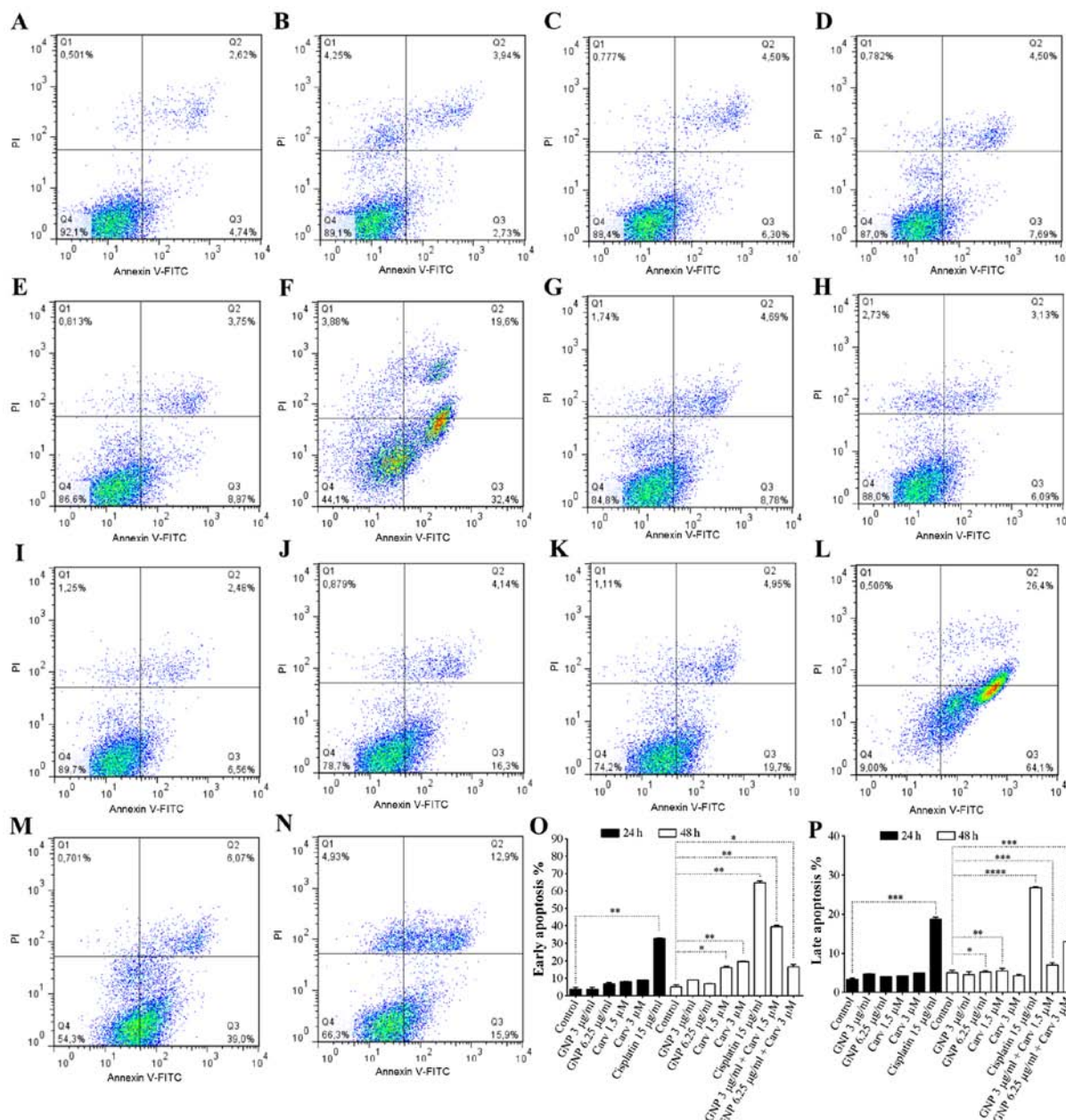


Figure 2. Flow cytometry to determine the cell death for apoptosis. Effect of different doses of GNPs, carvedilol, and cisplatin on early and late apoptosis in HepG2 cells. (A-F) At 24 h and (G-N) at 48 h. Control (A and G), GNPs 3 $\mu\text{g/ml}$ (B and H), GNPs 6.25 $\mu\text{g/ml}$ (C and I), Carv 1.5 μM (D and J), Carv 3 μM (E and K), cisplatin 15 $\mu\text{g/ml}$ (F and L). GNPs 3 $\mu\text{g/ml}$ + Carv 1.5 μM (M) and GNPs 6.25 $\mu\text{g/ml}$ + Carv 3 μM (N). (O) Early apoptosis and (P) late apoptosis. * $P<0.05$, ** $P<0.01$ and *** $P<0.001$.

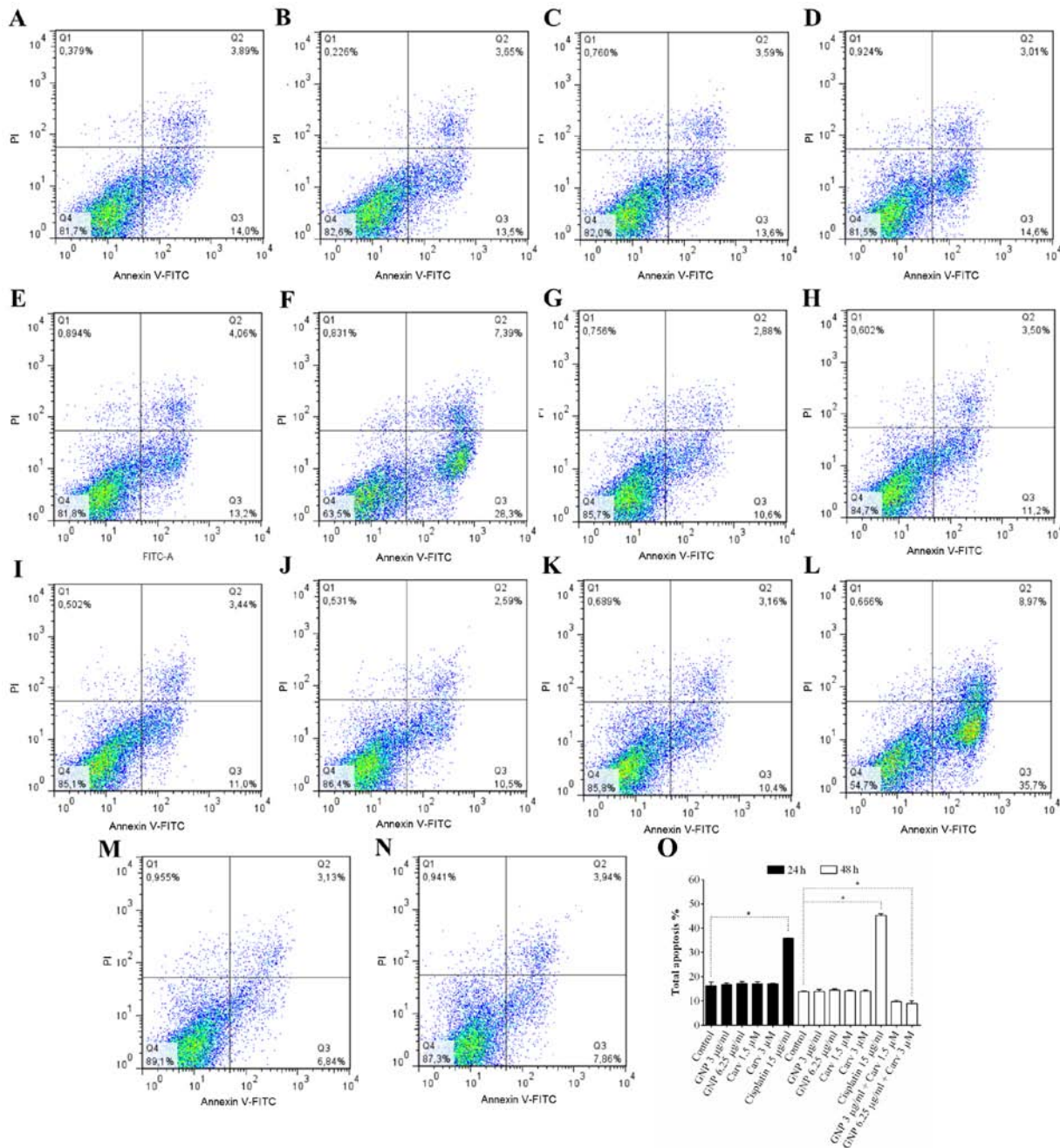


Figure 3. Flow cytometry to determine cell death by apoptosis. Effect of different doses of GNPs, carvedilol, and cisplatin on early and late apoptosis in HEK-293 cells at 24 h (A-F) and 48 h (G-N). Control (A and G), GNPs 3 µg/ml (B and H), GNPs 6.25 µg/ml (C and I), Carv 1.5 µM (D and J), Carv 3 µM (E and K), cisplatin 15 µg/ml (F and L). GNPs 3 µg/ml + Carv 1.5 µM (M) and GNPs 6.25 µg/ml + Carv 3 µM (N). (O) Total apoptosis. *P<0.05.

Results

Cell viability. The GNPs doses 1, 3 and 6.25 µg/ml, at 24 h (Fig. 1A) promoted low inhibition of cellular viability. The other GNPs doses promoted greater cellular growth inhibition, when compared to lower doses. The most significant was 12.5 µg/ml (P<0.05). At 48 h, GNPs doses of 3 and 6.25 µg/ml maintained low inhibition similar to that at 24 h (Fig. 1A), and for GNPs doses of 1, 25 and 50 µg/ml, there was cellular growth when compared to the same doses at 24 h. The carvedilol doses that promoted low inhibition of cellular viability were 1.5 and 3 µM at 24 h (Fig. 1B). The other carvedilol doses promoted greater cellular growth inhibition

(P<0.001). At 48 h, the 1.5 and 3 µM carvedilol doses maintained low inhibition (Fig. 1B). The other doses of carvedilol promoted greater cellular growth inhibition (P<0.001). DMSO at 1% was used as the carvedilol vehicle.

Detection of apoptosis, by flow cytometer. For HepG2 cells, combined treatment (GNP 6.25 µg/ml + carvedilol 3 µM) induced early (P<0.05) and late (P<0.001) apoptosis at 48 h (Fig. 2O-P). The isolated dose of carvedilol (3 µM) induced early apoptosis at 48 h (Fig. 2O; P<0.01). GNPs (6.25 µg/ml) induced late apoptosis at 48 h, P<0.05. Doses of GNPs and carvedilol did not induce statistically significant apoptosis in non-tumor cells (Fig. 3). Combined treatment

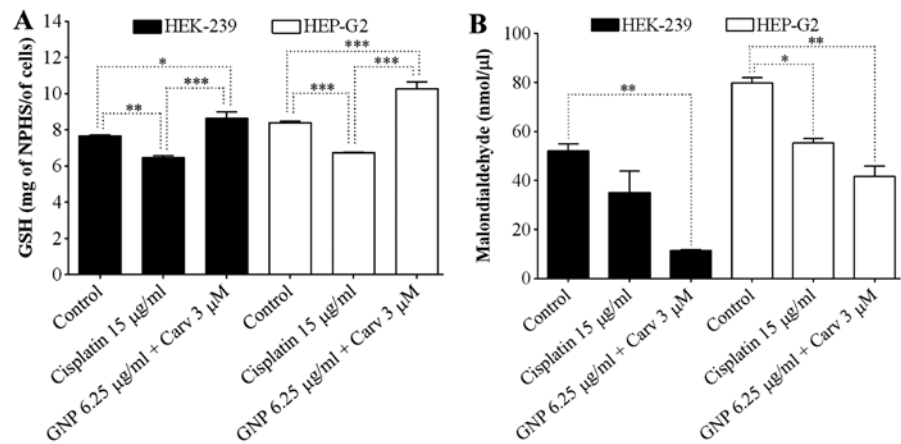


Figure 4. Oxidative stress markers. (A) GSH measurement, important protein in balance of oxidative stress. The combined treatment modulated GSH levels on HEK-293 (*P<0.05; **P<0.01 and ***P=0.0003) and HepG2 (*P<0.05; **P<0.01 and ***P<0.0001). (B) MDA measurement, indirect marker of oxidative stress. After combined treatment with GNPs and Carv, there was a reduction of the statistically significant levels of malondialdehyde in tumor and non-tumoral lines. *P<0.05; **P<0.01.

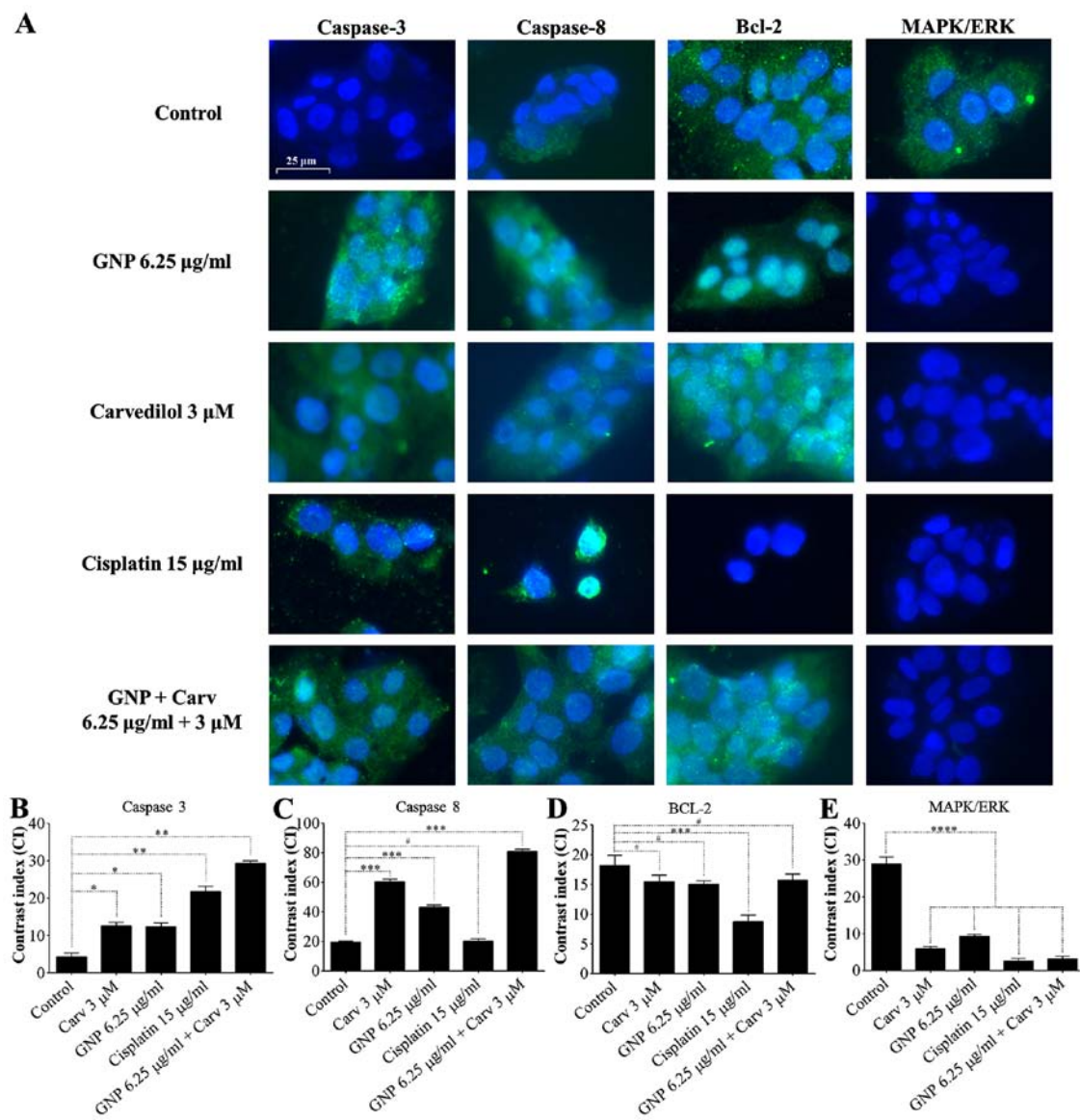


Figure 5. Detection of caspase-3, caspase-8, Bcl-2 and MAPK/ERK. HepG2 cells stained with DAPI (blue), anti-caspase-3, anti-caspase-8, anti-Bcl-2, and anti-MAPK/ERK antibodies (green) (A). Caspase-3 and caspase-8 were detected in all treated groups, yet MAPK/ERK was not. Bcl-2 did not alter expression. Contrast index for caspase-3, *P<0.05 and **P<0.01 (B); caspase-8, ***P<0.001 and #P>0.05 (C); Bcl-2, #P>0.05 and ***P<0.001 (D) and MAPK/ERK, ****P<0.0001 (E). All groups treated with GNPs + Carv showed high immunoreactivity for caspase-3, caspase-8 and low immunoreactivity for MAPK/ERK.

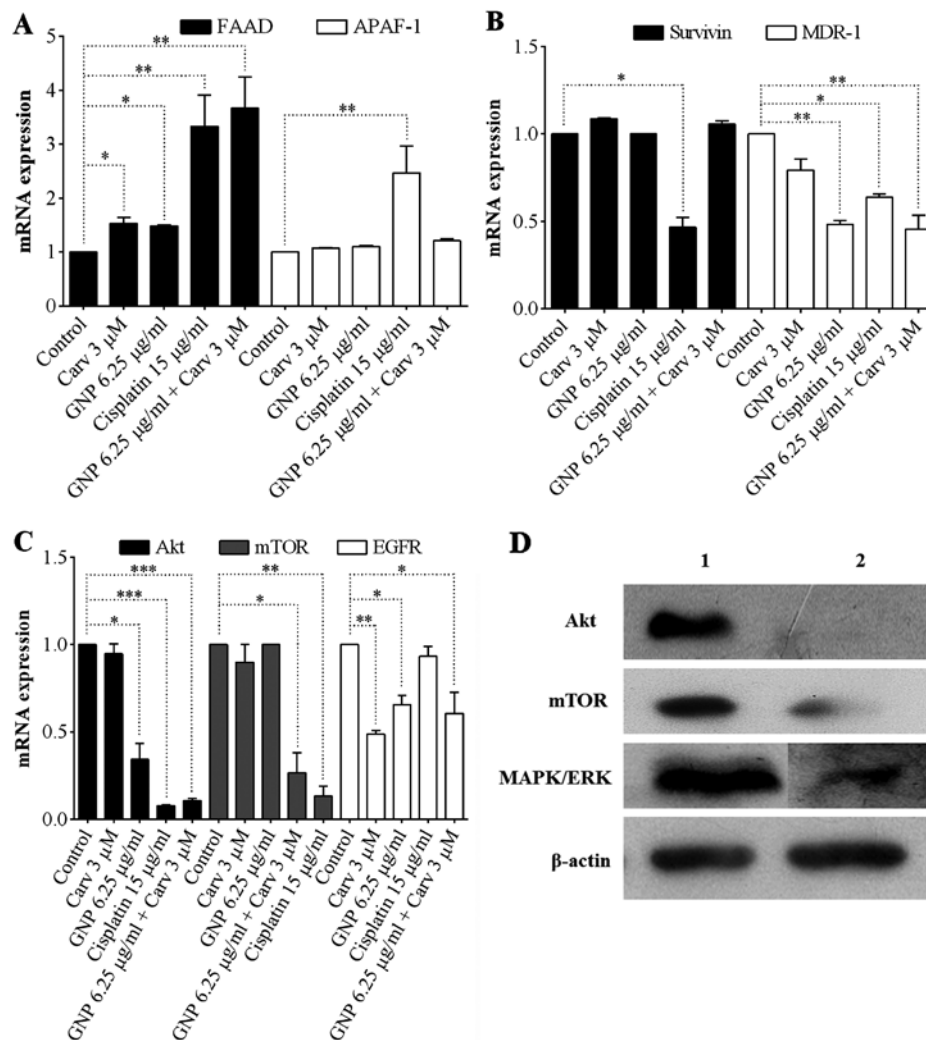


Figure 6. Gene and protein expression. Relative expression of genes related to survival and tumor resistance by real-time PCR. (A) FADD and Apaf-1, * $P < 0.05$ and ** $P < 0.01$; (B) survivin and MDR1, * $P < 0.05$ and ** $P < 0.01$; (C) EGFR, Akt and mTOR, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. (D) Western blot analysis of proteins related to survival and tumor proliferation. After exposure to the GNPs and carvedilol combination, there was a large decrease in the protein levels of Akt. mTOR and MAPK/ERK have a good reduction. β -actin was used as internal control. Lane 1, Control; lane 2, GNP 6.25 μ g/ml + Carv 3 μ M.

(GNP 6.25 μ g/ml + carvedilol 3 μ M) promoted cytoprotection ($P < 0.05$; Fig. 3O). When HepG2 and HEK-293 cells were treated with 15 μ g/ml cisplatin, apoptosis was detected at 24 and 48 h after treatment (Figs. 2F and L and 3F and L, respectively). The goal of using a non-tumor cell is to have as a control a non-tumoral lineage to analyze its behavior against the treatment. HEK-293 is used to study cytotoxicity because of its reliable growth despite its karyotype complex with alterations. This makes toxicity studies more consistent (54,58-62).

Oxidative stress. The reduced glutathione and lipid peroxidation levels were observed from GSH and MDA assays, respectively. Due to the fact that the combined treatment with GNPs (6.25 μ g/ml) + carvedilol (3 μ M), at 48 h increased the protection for HEK-293, it was observed that the levels of GSH increased statistically significantly for both HEK-293 ($P < 0.05$) and HepG2 ($P < 0.001$), as shown in Fig. 4A. Cisplatin promoted reduction of GSH levels in both cell lines. Regarding lipid peroxidation for the non-tumoral lineage, there was a great reduction in malondialdehyde levels using the combined treatment with GNPs (6.25 μ g/ml) + carvedilol (3 μ M) ($P < 0.01$),

overcoming the control and cisplatin treated groups. This same result was observed for the HepG2 tumor cells (Fig. 4B).

Immunofluorescence of caspase-3, caspase-8, Bcl-2 and MAPK/ERK. After combined treatment with GNPs (6.25 μ g/ml) + carvedilol (3 μ M), caspase-3 and caspase-8 marking was noted in all treated groups (Fig. 5A). Densitometric analysis confirmed significant increases in caspase-3 ($P < 0.01$; Fig. 5B), and caspase-8 ($P < 0.001$; Fig. 5C). In addition, there was no statistically significant change in Bcl-2 expression for the group treated with GNPs and carvedilol (Fig. 5D; $P > 0.05$). There was a decrease in MAPK/ERK immunoreactivity in the HepG2 cells treated with GNPs and carvedilol and GNP (6.25 μ g/ml) + carvedilol (3 μ M) for 48 h ($P < 0.0001$; Fig. 5E).

Gene and protein expression by RT-PCR and western blot analysis. From the gene expression analysis, it was observed that FADD elevation was statistically significant for all groups, those treated separately with GNPs (6.25 μ g/ml) and carvedilol (3 μ M) ($P < 0.05$), and in combined treatment GNPs (6.25 μ g/ml) + carvedilol (3 μ M) ($P < 0.01$), yet not observed

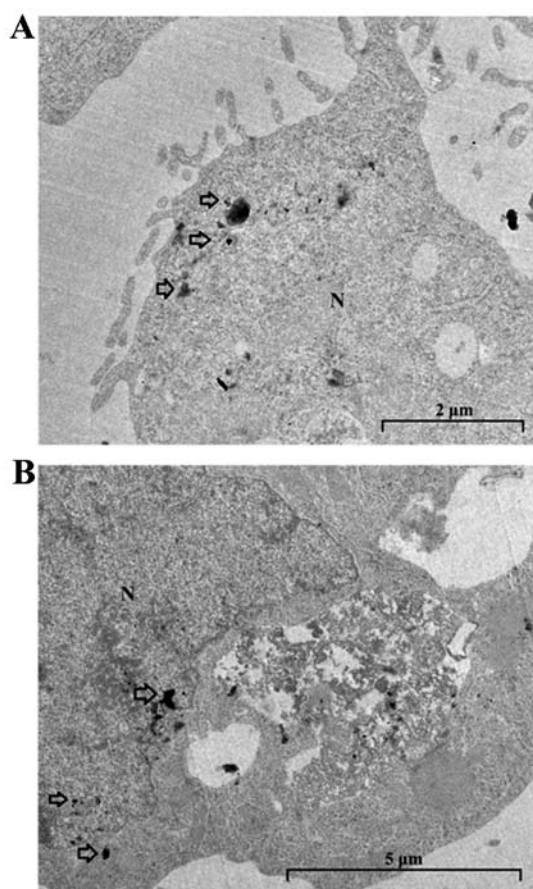


Figure 7. Transmission electron microscopy for evaluation of intracellular targets of treatment. (A) GNPs without carvedilol concentrated in the plasma membrane, indicated by black arrows. (B) For combined treatment, GNPs concentrated inside and beside the nucleus, indicated by black arrows. N, nucleus.

for APAF-1 levels (Fig. 6A). In relation to survivin levels, no group treated with GNPs and carvedilol demonstrated a reduction, meanwhile MDR-1 levels showed statistically significant reductions for those treated with GNPs (6.25 μg/ml), and for combined treatment GNPs (6.25 μg/ml) + carvedilol (3 μM) ($P < 0.01$; Fig. 6B). Regarding the Akt, mTOR and EGFR levels, the groups treated with combined treatment GNPs (6.25 μg/ml) + carvedilol (3 μM) showed statistically significant expression declines $P < 0.001$, $P < 0.05$ and $P < 0.05$, respectively (Fig. 6C). The groups treated with carvedilol (3 μM) showed statistically significant reductions only for EGFR ($P < 0.01$); and those treated with GNP showed statistically significant reductions only for Akt ($P < 0.05$) and EGFR ($P < 0.05$).

The anti-apoptotic protein levels after combined treatment of GNPs (6.25 μg/ml) + carvedilol (3 μM) are highlighted in Fig. 6D. The results showed a large reduction of Akt and mTOR. MAPK/ERK has a good reduced protein expression when compared to the control group. β-actin was used as internal control.

Transmission electron microscopy. TEM was performed in order to ascertain locations where gold nanoparticles would concentrate, applied without carvedilol (Fig. 7A) and in combination with carvedilol (Fig. 7B). Applied alone,

GNPs (6.25 μg/ml) was concentrated in the vicinity of the plasma membrane. In the combined treatment with GNPs (6.25 μg/ml) + carvedilol (3 μM), GNPs displayed intra-nuclear and perinuclear concentration.

Discussion

Data from the literature demonstrate the importance of using low doses for drug combination studies that aim to reduce side-effects and increase the field of action in different signaling pathways (63-65). In the present study, low doses of GNPs (3 and 6.25 μg/ml) and carvedilol (1.5 and 3 μM) were selected using the cell viability test, in order to use them in combination. Studies in the literature demonstrate that low doses of GNPs are more effective in inhibiting cell proliferation than larger doses (53,66). In relation to carvedilol, very high doses cause high inhibition of cell growth (67); a risk for a combined treatment. The pro-apoptotic activities of GNPs, carvedilol, and their combined use were analyzed by flow cytometry, both for tumor and non-tumoral lines. The combination of GNPs (6.25 μg/ml) + carvedilol (3 μM) promoted significant initial and late apoptosis in hepatic tumor cells, while promoting reduction of total apoptosis in the non-tumoral lineage. Studies in the literature report the ability of GNPs to induce apoptosis in several tumor cell lines without interfering with non-tumoral lineages (27,29,68,69). Carvedilol has itself also been shown to be an inducer of apoptosis in tumor cells and to protect normal cells (36,70,71).

The cytoprotective effect of the combined treatment for non-cancerous cells was evidenced through GSH dosage; an important balancing antioxidant system peptide (72,73). After treatment, GSH levels were elevated, which is important for reducing oxidative stress, which is related to apoptosis induction in several tissues (74-77). In addition, GNPs and carvedilol have the ability to promote elevation of GSH levels (78-81). Furthermore, a decrease in MDA levels were evidenced, an indirect biomarker for oxidative stress, as related to plasma membrane damage (82-84). This is a significant result, since one of the main side-effects of chemotherapy is lipid peroxidation (85,86).

In the tumoral lineage, we observed GSH level elevation and decreased MDA levels, demonstrating the antioxidant action of combined treatment when compared to cisplatin, which is known for its strong oxidative stress promotion (18,19). This result was highly positive, since tumor cells have high rates of reactive oxygen species, which trigger the activation of proteins such as Pi3K, Akt and MAPK/ERK; responsible for both cell proliferation and survival (87,88).

The pro-apoptotic activity of the treatment was confirmed by the positive immunoreactivity of caspase-3 and caspase-8, proteins involved in the extrinsic apoptosis pathway (89), these were the most significant in the group treated with the combination. In addition, after treatments with GNPs (6.25 μg/ml) + carvedilol (3 μM), there was a decrease in immunoreactivity for the MAPK/ERK, anti-apoptotic protein. A similar result is observed in western blot analysis. There were no significant changes for Bcl-2. These results indicate a prominent participation of the extrinsic apoptosis pathway, since Bcl-2 is an anti-apoptotic protein related to the intrinsic apoptosis pathway (mitochondrial) (54,90). GSH elevation and

MDA decreases, promoted by the combined treatment with GNPs (6.25 $\mu\text{g/ml}$) + carvedilol (3 μM), may have corroborated with mitochondria protection, justifying non-activation of the intrinsic apoptosis pathway (91-96). The statistically significant elevation of FADD, mainly by combined treatment with GNPs (6.25 $\mu\text{g/ml}$) + carvedilol (3 μM), confirms activation of the extrinsic apoptosis pathway, since it participates therein (97-99). Levels of APAF-1, a protein involved in the intrinsic pathway (100), did not present a significant differences.

The combined treatment with GNPs (6.25 $\mu\text{g/ml}$) + carvedilol (3 μM) was extremely effective on gene expression of anti-apoptotic proteins such as Akt and mTOR, matching or overcoming the action of cisplatin. Similar effect was observed in western blot analysis. Inhibition of these proteins, as well as MAPK/ERK, is critical for induction of apoptosis (12,51,52,101). Furthermore, other studies indicate that treatments with targeting of the Pi3K/Akt/mTOR pathway and the MAPK/ERK protein induce early and late apoptosis (102-104), as also obtained in the present study.

The decrease in EGFR levels is of great relevance since it is related to activation of Akt/mTOR and MAPK/ERK (105-108). However, it is possible that the combined treatment may be acting on EGFR-independent pathways that activate Akt and mTOR; and causing depletion (14,109-115). Levels of survivin were unchanged for treatment. However, in the same figure, there was a statistically significant reduction in MDR1 levels for both the combined treatment and the gold nanoparticles alone. Studies have shown that the use of nanoscale systems has excellent effects (this includes inorganic nanoparticles, such as gold nanoparticles), on the expression of genes related to multi-drug resistance, such as MDR1 (116,117). The decrease in the expression of this protein presents potential as a new strategy to combat one of the main problems of cancer treatment, resistance to treatment (118). Carvedilol, although showing no effect on MDR1 expression, has been reported in the literature as a potential MDR1 inhibitor (119,120). Studies in the literature have demonstrated that treatments acting on drug resistance genes promote both early and late apoptosis (121).

TEM did reveal GNPs internalization. However, we noted that when given alone, GNPs accumulated near the plasmatic membrane. Previous studies have reported that endosomal/lysosomal vesicles can imprison GNPs preventing complete action in the cellular interior, and are the greatest barrier that GNPs need to overcome to reach the cellular nucleus (main target) (122-125). However, after administration of carvedilol, concentration of GNPs was observed in the vicinity of the cell nucleus, both intra- and peripherally. The data possibly explain the fact that the results for GNPs administered alone show smaller indices as compared to the combined treatment, which was more effective. But even the isolated action of the gold nanoparticle can reduce, although less than combined treatment, the levels of survival and proliferation proteins such as Akt, EGFR, MDR-1 and MAPK/ERK, demonstrated in the present study, and suggest the way in which the GNPs inhibit proliferation. Concerning the combined treatment, carvedilol may be acting through other signaling pathways, or may be facilitating gold nanoparticle escape from endosomal/lysosomal vesicles. Han *et al* (126) reported that carvedilol has a role during receptor recycling in endosomal/lysosomal transiting of vascular smooth muscle cell beta-adrenergic receptors.

However, there remains the need for further chemical interaction and intracellular unfolding studies.

The present study demonstrated the ability of the combined GNPs and carvedilol treatment to induce apoptosis in the tumor cells by exclusively activating the extrinsic pathway. Activation of this pathway is advantageous compared to the intrinsic pathway, since it can be triggered independently of the p53 gene, which in many tumors is inactive or absent (127). In addition, studies have demonstrated that treatments with targeting intrinsic pathway induction may also promote positive selection of tumor cells, while evading the mitochondrial pathway (128). In addition to these results, the combined treatment protected non-tumor cells, reducing oxidative stress and consequently, apoptosis. The novel findings in these studies highlight a promising alternative for future cancer treatments.

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