Metformin in combination with chemotherapy increases apoptosis in gastric cancer cells and counteracts senescence induced by chemotherapy

KATIA CAROLINA VÁZQUEZ-IBARRA[1], JOSEFINA YOALY SÁNCHEZ LÓPEZ[2], TOMÁS DANIEL PINEDA RAZO[3],
JOSÉ ROBERTO CRUZ LOZANO[4], BRENDA GUADALUPE ORTIZ-TAMAYO[5],
LUIS ARTURO PALAFOX-MARISCAL[4], ROSA MARÍA GONZÁLEZ ARREOLA[1],
JUAN RAMÓN GONZÁLEZ-GARCÍA[2] and PABLO CESAR ORTIZ-LAZARENO[6]

[1]Department of Molecular Biology and Genomics, University Center for Health Sciences, University of Guadalajara,
Guadalajara, Jalisco 44340, Mexico; [2]Genetic Division, Western Biomedical Research Center, Mexican Social Security Institute,
Guadalajara, Jalisco, 44340, Mexico; [3]Medical Oncology Service, Western National Medical Center, Mexican Social Security Institute,
Guadalajara, Jalisco 44329, Mexico; [4]Department of Physiology, University Center for Health Sciences, University of Guadalajara,
Guadalajara, Jalisco 44340, Mexico; [5]Division of Biological and Environmental Sciences, University Center of Biological and
Agricultural Sciences, University of Guadalajara, Guadalajara, Jalisco 44600, Mexico; [6]Immunology Division,
Western Biomedical Research Center, Mexican Social Security Institute, Guadalajara, Jalisco 44340, Mexico

Abstract. Gastric cancer (GC) is the fourth leading cause of cancer death in the world, and there is a demand for new therapeutic agents to treat GC. Metformin has been demonstrated to be an antineoplastic agent in some types of cancer; however, it has not been sufficiently valued in treating GC because the effect of metformin in combination with chemotherapy regimens has not yet been evaluated. The present study aimed to evaluate the mechanisms underlying cell death induced by metformin alone or when combined with chemotherapy. The cytogenetic characteristics of the NCI-N87 cell line were determined by fluorescence *in situ* hybridization (FISH). To determine viability, the cells were treated with metformin, epirubicin, cisplatin, docetaxel and 5-fluorouracil (individually and at different concentrations). Subsequently, the cells were treated with metformin alone, and in combination with the chemotherapeutic drugs and the epirubicin + cisplatin + 5-fluorouracil, docetaxel + cisplatin + 5-fluorouracil, and cisplatin + 5-fluorouracil regimens. Cell viability, proliferation and mitochondrial membrane potential ($\Delta\Psi$m) were analyzed by spectrophotometry. Apoptosis, caspase activity and cell cycle progression were assessed by flow cytometry. Finally, light microscopy was used to evaluate senescence and clonogenicity. The results revealed that metformin, alone and when combined with chemotherapy, increased the proportion of apoptotic cells, promoted the loss of $\Delta\Psi$m, and induced apoptosis through caspase activity in GC cells. Moreover, metformin decreased cell proliferation. In addition, metformin alone did not induce senescence and it counteracted the effects of chemotherapy-induced senescence in GC cells. Additionally, metformin, alone and when combined with chemotherapy, decreased the clonogenic capacity of NCI-N87 GC cells. In conclusion, metformin may increase the effects of chemotherapy on NCI-N87 cell death and could represent an option to improve the treatment of GC.

Introduction

Gastric cancer (GC) is the fourth leading cause of cancer death worldwide, and 1,089,103 new cases and 768,793 deaths were reported in 2020 (1). The incidence of GC varies by region; with the highest prevalence detected in eastern and central Asia, and Latin America (2). Notably, this type of cancer has no symptoms in the early stages and patients diagnosed at advanced stages have unfavorable prognoses (3,4). GC survival varies depending on the stage of the disease during surgical intervention; for early advanced stages, the 5-year survival rate is 18% (5). Due to the poor prognosis of GC, its therapeutic resistance and the side effects of chemotherapy, it is essential to develop new strategies for the treatment of GC.

Metformin has attracted attention as an antitumor agent that may be used as an alternative treatment to improve the survival and quality of life of patients with GC (6,7). Metformin (1,1 dimethylbiguanide) is an antihyperglycemic drug used to treat diabetes mellitus type 2 (DM2) (8). Epidemiological, *in vitro* and *in vivo* studies, and clinical trials have demonstrated the ability of metformin as an antitumor agent in various types of cancer, such as lung, endometrial, breast and prostate cancer (9,10). The antitumor mechanism of action of metformin is not fully understood; however, two mechanisms have been proposed: A direct effect through activating AMPK, and an indirect effect by reducing blood glucose and insulin levels (9). Metformin is internalized into cells primarily by organic cation transporter 1 (11). Once inside the cell, metformin acts directly on the mitochondria, inhibiting the first complex of the electron transport chain (NADH dehydrogenase). This process decreases the amount of ATP and increases the AMP/ATP ratio. An increased concentration of cytosolic AMP leads to AMPK activation, and liver kinase B1 is the enzyme that activates AMPK via phosphorylation of the Thr172 residue of the α subunit (12). Subsequently, the metabolism of the cell changes from an anabolic state to a catabolic state to restore cellular homeostasis; therefore, processes such as gluconeogenesis, lipogenesis and protein synthesis are inhibited (13).

Previous studies have demonstrated that the antitumor effect of metformin alone on GC cell lines is capable of decreasing cell viability (14-17), cell proliferation (14,15,17) and cell survival (17), and increasing apoptosis (15,16,18). In addition, *in vivo* models of metformin have shown its potential antitumor effect reducing the expression of cyclin D, cyclin-dependent kinase (CDK)4 and CDK6, and the levels of retinoblastoma protein (Rb) phosphorylation (14), as well as inhibiting survivin and mTOR (18).

To the best of our knowledge, the effect of metformin in combination with chemotherapeutic drugs has yet to be sufficiently studied; cisplatin, adriamycin, paclitaxel, oxaliplatin, docetaxel, 5-fluorouracil, rapamycin, carboplatin, epirubicin or methotrexate in combination with metformin have been analyzed for the treatment of GC *in vitro* (17,19-22). However, the combination of metformin with chemotherapy regimens has not been carried out for GC treatment, which involves the combination of different drugs and the activation of multiple pathways, including apoptosis, proliferation and resistance. The present study aimed to evaluate the mechanisms underlying the effects of metformin, alone or in combination with chemotherapy, on the apoptosis, mitochondrial membrane potential (ΔΨm), caspase-3, -8 and -9 activity, cell cycle progression, proliferation, senescence and clonogenic capacity of NCI-N87 GC cells.

Materials and methods

Cell culture and reagents. The NCI-N87 GC cell line was obtained form from American Type Culture Collection (CRL-5822™). Cells were maintained in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% inactivated fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.) and 1% penicillin-streptomycin-neomycin (PSN; Gibco; Thermo Fisher Scientific, Inc.) at 37°C in a humidified atmosphere containing 95% air and 5% CO_2. The cells were passaged once they reached 75-85% confluence. Before the initiation of all experiments, cell viability was determined with Trypan Blue (MilliporeSigma) and viability was >95%. This study was approved by the National Scientific Research Committee of Mexican Social Security Institute (approval number: R-2019-785-050; Guadalajara, Mexico).

Cytogenetic characterization of the GC cell line. The cytogenetic characteristics of NCI-N87 cells were assessed by fluorescence *in situ* hybridization (FISH) using commercially available direct labeled FISH probes. NCI-N87 cells were harvested using Accutase (cat. no. 423201; Biolegend, Inc.) and were washed twice with PBS. The cells were then resuspended in 1 ml RPMI-1640 supplemented with 10% inactivated FBS and 1% PSN, and those harvested cells were treated with 0.075 M potassium chloride solution at 37°C for 20 min, centrifuged at 240 x g for 10 min at 25°C, fixed in methanol:acetic acid (3:1) solution at 2°C, resuspended in the same fixing solution, and dropped onto cleaned microscope slides for FISH. The cells were dehydrated and hybridized according to the FISH probe manufacturer's protocol. The following probes were obtained from Cytocell (Oxford Gene Technology IP Limited): *DXZ1/DYZ3* (cat no. LPE 0XY), *CKS1B/CDKN2C* (cat. no. LPH 039), *EGFR* amplification (cat. no. LPS 003), *MYC* breakapart (cat. no. LPH 010), *CDKN2A-B/D9Z3* (cat. no. LPH 009), *IGH/CCND1* (cat. no. LPH 072), *RB1/LAMP1* (cat. no. LPS 011), *IGH/BCL2* (cat. no. LPH 071), *TP53/ATM* (cat. no. LPH 052), *ERBB2/D17Z1* (cat. no. LPS 001), *PML/RARA* (cat. no. LPH 023) and *TCRAD* breakapart (cat no. LPH 047). Samples were observed under a fluorescence microscope and microscopic observations were interpreted following the International System for Human Cytogenomic Nomenclature 2020 recommendations (23). Only FISH assays with abnormal results are presented in the present study.

Drugs. Before performing the experiments in GC cells, various solutions were prepared. Metformin (cat. no. 317240; Merck KGaA) was dissolved in RPMI and stored at -20°C until use. Epirubicin (cat. no. E9406; MilliporeSigma), 5-fluorouracil (cat. no. F6627; MilliporeSigma) and cisplatin (cat. no. P4394; MilliporeSigma) were dissolved in sterile saline and maintained at 4°C, with the exception of cisplatin, which was stored at room temperature. Docetaxel (cat. no. 01885; MilliporeSigma) was dissolved in DMSO and stored at -80°C.

Cell treatments. NCI-N87 cells were treated for 48 h at 37°C with metformin (10 mM) and four chemotherapy drugs: epirubicin (0.5 μM), cisplatin (15 μM), docetaxel (0.5 μM) or 5-fluorouracil (30 μM). In addition, metformin was used in combination with each of the chemotherapy drugs, as well as with the three chemotherapy regimens: Epirubicin (0.5 μM) + cisplatin (15 μM) + 5-fluorouracil (30 μM) (ECF), docetaxel (0.5 μM) + cisplatin (15 μM) + 5-fluorouracil (30 μM) (DCF) and cisplatin (15 μM) + 5-fluorouracil (30 μM) (CF). The control group consisted of cells without treatment.

Determination of apoptosis in GC cells. NCI-N87 cells ($5x10^5$ cells/well) were seeded in 24-well plates and cultured in 1 ml RPMI-1640 supplemented with 10% inactivated FBS

and 1% PSN for apoptosis determination. The next day, the seeded cells were treated with the drugs for 48 h at 37°C. Subsequently, cells were harvested using Accutase and were washed twice with PBS. The cells were then resuspended in 400 μl Annexin V Binding Buffer, and FITC Annexin V (cat. no. 640922; Biolegend) and SYTOX® (cat. no. S34859; Invitrogen, Carlsbad, CA, USA) were added. The cells were incubated for 15 min in the dark at 25°C. Finally, 10,000 events were acquired for each sample using the Attune Acoustic Focusing Cytometer (Applied Biosystems; Thermo Fisher Scientific, Inc.). The data were analyzed using Kaluza V2.1 software (Beckman Coulter, Inc.). The results of apoptosis analysis were expressed as the mean ± SD of live, apoptotic and necrotic cells. Etoposide (100 μM) (PiSA Farmacéutica) was used as a positive control for the cell death assay (data not shown).

Determination of ΔΨm in GC cells. The loss of ΔΨm was determined using JC-10 reagent (cat. no. ab112134; Abcam). NCI-N87 cells (4×10^4 cells/well) were seeded in 96-well plates with black wells and clear bottoms and were cultured in 100 μl RPMI-1640 supplemented with 10% inactivated FBS and 1% PSN. The next day, cells were exposed to the different treatments for 48 h. For JC-10 staining, 50 μl JC-10 was diluted in 5 ml Assay Buffer A, and 50 μl of the mix was added to each sample and incubated at 37°C for 1 h. Subsequently, 50 μl Assay Buffer B was added before measuring fluorescence intensity. Finally, fluorescence was measured at excitation 488 nm and emission ratio 530/590 nm in a plate reader (Biotek Synergy HT; Biotek; Agilent Technologies, Inc.). Data are presented as the mean ± SD. The positive control for the ΔΨm assay was etoposide (100 μM) (data not shown).

Determination of caspase activity in GC cells. To evaluate activated caspase-1, -3, -4, -5, -6, -7, -8 and -9 in apoptotic cells, a Generic Caspase Activity Assay kit (cat. no. ab112130; Abcam) was used. NCI-N87 cells (5×10^5 cells/well) were seeded in 24-well plates and cultured in 1 ml RPMI-1640 supplemented with 10% inactivated FBS and 1% PSN. The next day, cells were exposed to different treatments for 48 h. Cells were harvested using Accutase and were washed twice with PBS. Then, cells were resuspended in 0.5 ml culture medium, and 1 μl TF2-VAD-FMK (obtained from the Generic Caspase Activity Assay kit) was added to each sample and the cells were incubated at 37°C for 2 h. Subsequently, the cells were washed once with PBS. Finally, cells were resuspended in 0.5 ml Assay Buffer. A total of 10,000 events were acquired for each sample using the Attune Acoustic Focusing Cytometer. The data were analyzed using Kaluza V2.1 software. Data are presented as the mean ± SD of cell percentage. The positive control for the general caspase activity assay was etoposide (100 μM) (data not shown).

Determination of caspase-3, -8 and -9 activity in GC cells. To evaluate the activity of caspases, the NCI-N87 cells (5×10^5 cells/well) were seeded in 24-well plates and cultured in 1 ml RPMI-1640 supplemented with 10% inactivated FBS and 1% PSN. The next day, cells were exposed to different treatments for 48 h. Three different kits were used to determine the activity of each caspase (caspase-3, caspase-8 and caspase-9,

cat. no. ab65613, ab65614 and ab65615, respectively; Abcam). Cells were harvested using Accutase and were washed twice with PBS. Then, cells were resuspended in 300 μl culture medium, and 1 μl of the corresponding substrate was added (FITC-DEVD-FMK/caspase-3, FITC-IETD-FMK/caspase-8 and FITC-LEHD-FMK/caspase-9) for 1 h at 37°C. Subsequently, the cells were washed once with 500 μl Wash Buffer, the supernatant was removed and the cells were resuspended in 300 μl Wash Buffer. At least 10,000 events were acquired using the Attune Acoustic Focusing Cytometer and the data were analyzed using Kaluza V2.1 software. Data are presented as the mean ± SD of cell percentage. The positive control for the caspase activity assay was etoposide (100 μM) (data not shown).

Cell cycle assessment in GC cells. Cell cycle progression was determined using the BD Cycletest™ Plus DNA kit (cat. no. 340242; BD Biosciences). To synchronize the cells, they were depleted of serum in a step-by-step manner: Cells were cultured with RPMI-1640 supplemented with 5% FBS for 12 h; after which, cells were cultured with RPMI-1640 supplemented with 1% FBS for 12 h; finally, cells were cultured with serum-free RPMI-1640 for 18 h. After synchronization, NCI-N87 cells (5×10^5 cells/well) were seeded in 24-well plates and cultured in 1 ml RPMI-1640 supplemented with 10% inactivated FBS and 1% PSN. The next day, seeded cells were treated with different drugs and combinations for 48 h. Subsequently, the cells were harvested using Accutase and were washed twice with PBS, before the DNA staining procedure was performed. First, trypsin buffer was added to each sample and incubated at room temperature for 10 min; after which, a trypsin inhibitor and RNase buffer were added and incubated for 10 min at room temperature. Finally, propidium iodide solution was added and incubated on ice for 10 min in the dark. At least 30,000 events were acquired for each sample using the Attune Acoustic Focusing Cytometer. Data were analyzed using ModFit LT 5.0 software (Verity Software House, Inc.). Data are presented as the mean ± SD of the percentage of cells in the G_1, S and G_2 phases. The DNA QC particles kit (cat. no. 349523; BD Biosciences) was used to check the calibration and linearity of the equipment (data not shown).

Proliferation assay in GC cells. Proliferation was determined using the BrdU Cell Proliferation ELISA Kit (cat. no. ab126556; Abcam). The NCI-N87 cells (4×10^4 cells/well) were seeded in 96-well plates and cultured in 200 μl RPMI-1640 supplemented with 10% inactivated FBS and 1% PSN. The next day, cells were exposed to the different treatments for 96 h, and BrdU was incubated for 24 h at 37°C. The cell culture medium was aspirated, 200 μl/well fixing solution was added to denature the DNA and the cells were incubated for 30 min at room temperature. Subsequently, the first wash was performed using Wash Buffer; after which, 100 μl/well Anti-BrdU Monoclonal Detector Antibody was added and the cells were incubated for 1 h at room temperature. A second wash was then performed and 100 μl/well Peroxidase Goat Anti-Mouse IgG Conjugate was used to incubate the cells for 30 min at room temperature. Subsequently, a third wash was performed, 100 μl/well TMB Peroxidase Substrate was added and the cells were incubated for 30 min at room temperature in

the dark. Finally, 100 μl/well Stop Solution was added and the optical density was determined at 450 nm using a plate reader (Biotek Synergy HT). Data are presented as the mean ± SD of the percentage of proliferation. The positive control for the cell proliferation assay was etoposide (100 μM) (data not shown).

Senescence assessment in GC cells. Senescence was evaluated using the Senescence Detection Kit (cat. no. ab65351; Abcam). The NCI-N87 cells (1x10^6 cells/well) were seeded in 12-well plates and cultured in 2 ml RPMI-1640 supplemented with 10% inactivated FBS and 1% PSN. The next day, seeded cells were treated with the different drugs for 48 h. The culture medium was then removed and the cells were washed once with 1 ml PBS. Subsequently, the cells were fixed with 0.5 ml Fixative Solution for 15 min at room temperature, washed twice with PBS, and incubated with 0.5 ml Staining Solution Mix (Staining Solution, Staining Supplement and β-galactosidase) inside a sealable bag overnight at 37°C. Cells were observed under a light microscope to determine senescent cells. Data were analyzed using ImageJ software (Version 1.8.0_172; National Institutes of Health). Data are presented as the mean ± SD of β-galactosidase-stained surface. The positive control for the senescence assay was doxorubicin (1 μM) (PiSA Farmacéutica) (data not shown).

Clonogenic assay in GC cells. Cell survival was determined using a clonogenic assay. The NCI-N87 cells (15x10^5 cells/well) were seeded in 6-well plates and cultured in 3 ml RMPI-1640 supplemented with 10% inactivated FBS and 1% PSN. The following day, cells were exposed to the different treatments for 24 h. Subsequently, the cells were harvested using Accutase and were washed twice with PBS. Then, 4,000 cells/well were seeded in 6-well plates in 3 ml culture medium. To determine the ability to form colonies, the cells were incubated for 15 days at 37°C (during this period, the culture medium was changed every 3 days). To stain colonies, the cells were first fixed with 1 ml/well formaldehyde (3.7% diluted in PBS) for 15 min at 25°C, washed twice with 2 ml PBS and dried overnight. Colonies were stained with 1 ml/well sulforhodamine (0.4% diluted in 1% acetic acid) for 30 min at 25°C and were finally washed three times with acetic acid (1% diluted in H$_2$O). Colonies (>60 cells) were viewed under a light microscope at x40 magnification and images were captured using Zen 2012 blue edition v1.1.2.0 software (Zeiss GmbH). The colony count was performed with ImageJ software. The positive control for the clonogenic assay was etoposide (100 μM) (data not shown).

Statistical analysis. All data are presented as the mean ± SD of three independent experiments performed in triplicate. To assess normality, the Shapiro-Wilk test was performed. Two-way ANOVA was used for statistical analysis, followed by Tukey post hoc test to compare all cell treatments. $P < 0.05$ was considered to indicate a statistically significant difference. Data were analyzed using GraphPad Prism v8.0.2 software (Dotmatics).

Results

Treatment with metformin alone, and in combination with chemotherapy, increases apoptosis and promotes loss of ΔΨm in NCI-N87 GC cells. The results of the present study

showed that metformin alone induced apoptosis compared with in the untreated cells control ($P < 0.05$; Figs. 1A and S1B). Furthermore, epirubicin, cisplatin, and 5-fluorouracil increased the percentage of apoptotic cells ($P < 0.05$), and when metformin was combined with each chemotherapy drug, it was observed that the tendency was for it to improve the effectiveness of chemotherapy drug-induced apoptosis. However, only the combination of metformin + 5-fluorouracil was significant compared with 5-fluorouracil alone ($P < 0.05$).

The chemotherapy regimens ECF, DCF and CF induced the apoptosis of NCI-N87 cells ($P < 0.05$), and the combination of metformin with the ECF regimen significantly enhanced the apoptosis of GC cells compared with the regimen alone ($P < 0.05$) (Figs. 1B and S1C).

Notably, metformin alone significantly induced a loss of ΔΨm in GC cells ($P < 0.05$), as did the four chemotherapeutic drugs ($P < 0.05$), when compared with the control group (Fig. 1C). When metformin was combined with each of the chemotherapeutic drugs, a greater effect on the loss of ΔΨm in GC cells was observed ($P < 0.05$) compared with chemotherapy drugs alone. In addition, the ECF, DCF and CF chemotherapy regimens decreased the ΔΨm of NCI-N87 cells ($P < 0.05$), and when metformin was combined with DCF and CF regimens, that effect was amplified in comparison with the chemotherapy regimens alone ($P < 0.05$) (Fig. 1D).

Apoptosis is induced through caspase activity. Metformin increased caspase activity in comparison with untreated cells ($P < 0.05$; Figs. S2 and 2A). In addition, the four chemotherapy drugs alone significantly increased caspase activity compared with that in the control group ($P < 0.05$). Additionally, the combinations of metformin + epirubicin and metformin + 5-fluorouracil induced significantly increased caspase activity compared with that in the cells treated with chemotherapy drugs alone ($P < 0.05$). By contrast, the combination of metformin + cisplatin and metformin + docetaxel had no significant effect.

The chemotherapy regimens ECF, DCF and CF also increased caspase activity compared with that in the control group ($P < 0.05$); however, when metformin was combined with each chemotherapy regimen, there was no significant difference compared with the regimen alone (Fig. 2B).

After confirming that all treatments induced caspase activity, the present study further investigated the participation of the executioner caspase-3 and the initiator caspases-8 and -9. Metformin alone increased the activities of caspases-3, -8 and -9 in GC cells compared with those in the control group ($P < 0.05$; Fig. 2C-H). In addition, epirubicin, cisplatin and 5-fluorouracil increased the activities of the three caspases ($P < 0.05$), whereas docetaxel only significantly increased caspase-9 activity ($P < 0.05$). Metformin in combination with cisplatin and 5-fluorouracil increased caspase-3 activity ($P < 0.05$) compared with each drug alone. Whereas metformin in combination with all chemotherapy drugs increased caspase-8 and -9 activities compared with the chemotherapy drugs alone ($P < 0.05$). These findings indicated that metformin may enhance the effectiveness of chemotherapeutic drugs by increasing caspase activity.

The chemotherapy regimens ECF, DCF and CF increased the activities of caspases-3, -8 and -9 compared with that in the control group ($P < 0.05$; Fig. 2D, F and H). In addition, metformin
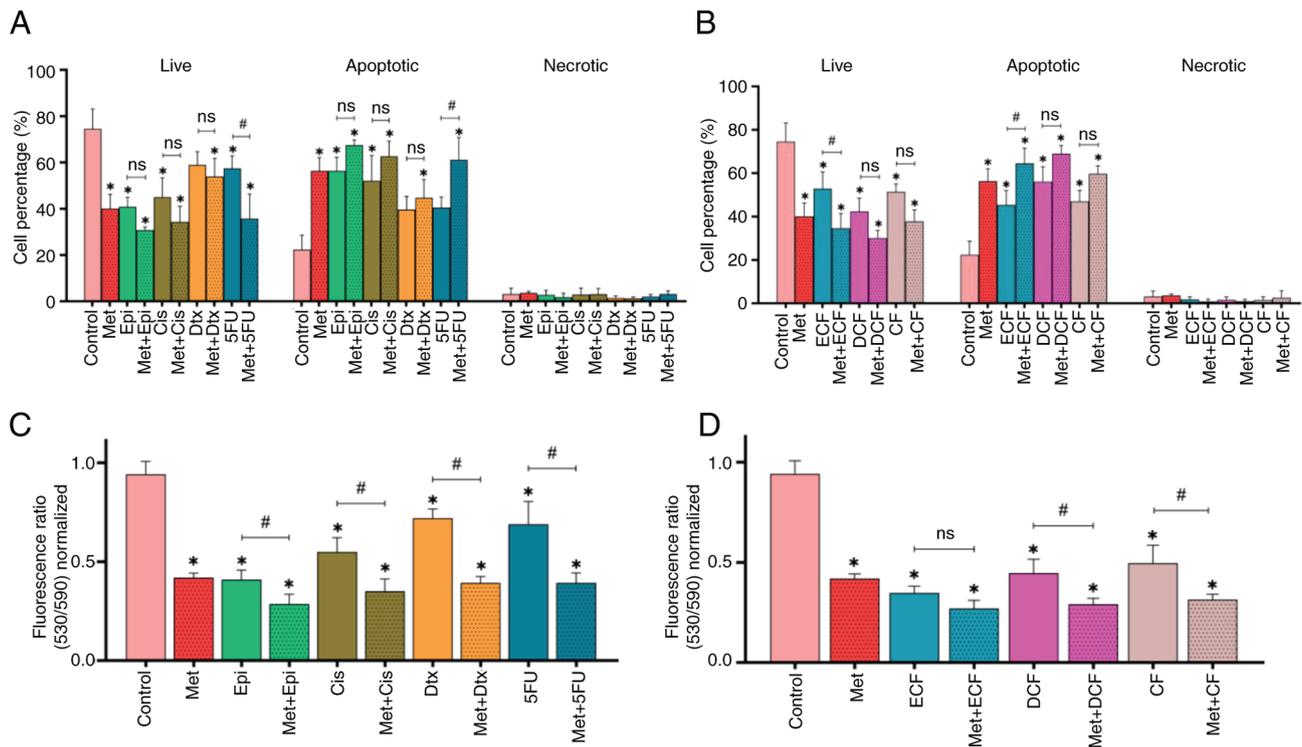
Figure 1. Effect of Met in combination with chemotherapy on apoptosis and loss of mitochondrial membrane potential. Percentage of live, apoptotic and necrotic cells treated with Met in combination with (A) chemotherapy drugs and (B) chemotherapy regimens. Loss of mitochondrial membrane potential of NCI-N87 gastric cancer cells treated with Met in combination with (C) chemotherapy drugs and (D) chemotherapy regimens. Data are presented as the mean ± SD from three independent experiments performed in triplicate. *P<0.05 vs. control; #P<0.05. Met, metformin; Epi, epirubicin; Cis, cisplatin; Dtx, docetaxel; 5FU, 5-fluorouracil; ECF, epirubicin + cisplatin + 5-fluorouracil; DCF, docetaxel + cisplatin + 5-fluorouracil; CF, cisplatin + 5-fluorouracil; ns, not significant.

in combination with DCF and CF increased caspase-3, -8, and -9 activity compared with the regimens alone (P<0.05). By contrast, metformin combined with ECF only significant increased caspase-9 activity compared to the ECF regimen alone (P<0.05).

Cell cycle progression in NCI-N87 GC cells treated with metformin alone and in combination with chemotherapy drugs. Most NCI-N87 cells treated with metformin were accumulated in the G_0/G_1 phase of the cell cycle (50.31%) (Fig. 3A and B). Epirubicin and cisplatin are agents non-specific to the cell cycle phase, which can affect cells in all cell cycle phases (24). It was observed that most GC cells accumulated in the S phase of the cell cycle when they were treated with epirubicin (53.08%) or cisplatin (47.82%). On the other hand, docetaxel and 5-fluoro-uracil are cell cycle-specific agents, which act in one particular phase of the cell cycle (G_2/M and S, respectively) (24). Most of the GC cells treated with docetaxel were in the G_2/M phase (69.53%), and cells treated with 5-fluorouracil accumulated in both G_0/G_1 (45.73%) and S phases (41.87%).

GC cells exposed to chemotherapy regimens ECF and CF accumulated in the G_0/G_1 phase (56.9 and 51.9%, respectively), whereas cells treated with DCF accumulated in both the G_0/G_1 (40.2%) and G_2/M phases (31.7%) (Fig. S3).

Metformin alone decreases the proliferation of NCI-N87 GC cells. Some genes involved in different pathways that promote cancer progression were assessed in the present study. The results revealed that NCI-N87 cells showed

deletion of *BCL2* and *TP53* genes (Fig. 4A and B), indicating that other proteins may participate in the apoptosis process, caspase pathways and proliferation. Notably, uncontrolled proliferation is a hallmark of cancer cells. NCI-N87 GC cells showed amplification of *ERBB2* and duplication of *MYC* (Fig. 4C and D), which may favor the proliferation of these cells. Therefore, it was considered essential to evaluate the effect of metformin on cell proliferation. NCI-N87 GC cells were exposed to the treatments for 96 h. The results revealed that metformin alone significantly reduced the proliferation of GC cells compared with that in the control group (P<0.05; Fig. 4E). Similarly, epirubicin, cisplatin, docetaxel and 5-fluorouracil exhibited an antiproliferative effect on GC cells (P<0.05). Notably, the combination of metformin with each chemotherapy drug did not significantly affect cell proliferation compared with the chemotherapy drugs alone. Similar effects were observed in response to chemotherapy regimens. Treatments with ECF, DCF and CF significantly reduced cell proliferation compared with that in the control group (P<0.05), whereas no differences were observed when metformin was combined with each chemotherapy regimen (Fig. 4F).

Metformin alone does not induce senescence and counteracts the effect of chemotherapy-induced senescence in NCI-N87 GC cells. The present study demonstrated that metformin did not induce senescence; however, epirubicin, cisplatin and 5-fluorouracil alone significantly induced senescence compared
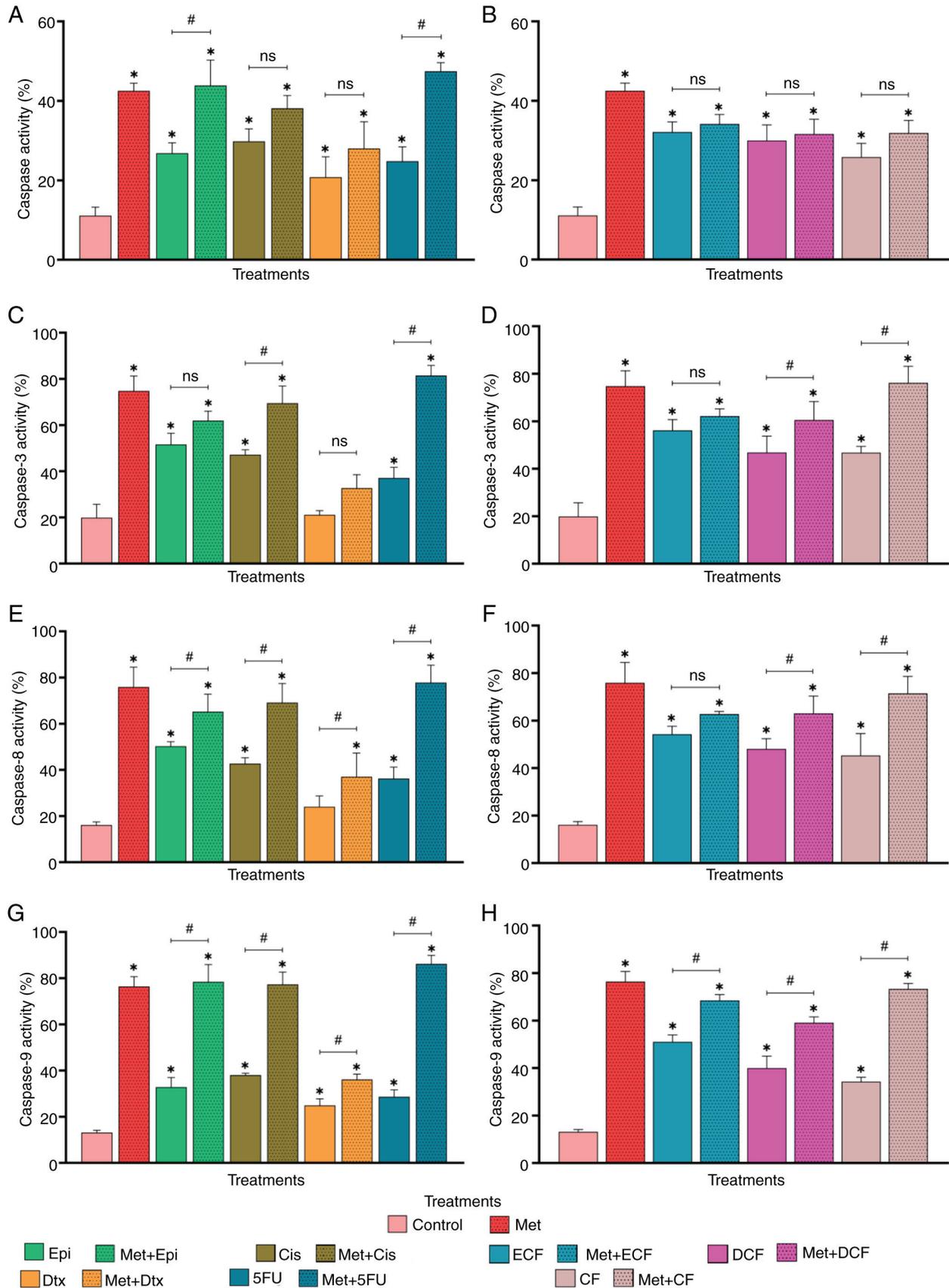
Figure 2. Effect of Met in combination with chemotherapy drugs on caspase activity. Percentage of caspase-1, -3, -4, -5, -6, -7, -8, and -9 activity (TF2-VAD-FMK) in cells treated with Met in combination with (A) chemotherapy drugs and (B) chemotherapy regimens. Percentage of caspase-3 activity (FITC-DEVD-FMK) in cells treated with Met in combination with (C) chemotherapy drugs and (D) chemotherapy regimens. Percentage of caspase-8 activity (FITC-IETD-FMK) in cells treated with Met in combination with (E) chemotherapy drugs and (F) chemotherapy regimens. Percentage of caspase-9 activity (FITC-LEHD-FMK) in cells treated with Met in combination with (G) chemotherapy drugs and (H) chemotherapy regimens. Data are presented as the mean ± SD from three independent experiments performed in triplicate. *$P<0.05$ vs. control; #$P<0.05$. Met, metformin; Epi, epirubicin; Cis, cisplatin; Dtx, docetaxel; 5FU, 5-fluorouracil; ECF, epirubicin + cisplatin + 5-fluorouracil; DCF, docetaxel + cisplatin + 5-fluorouracil; CF, cisplatin + 5-fluorouracil; ns, not significant.
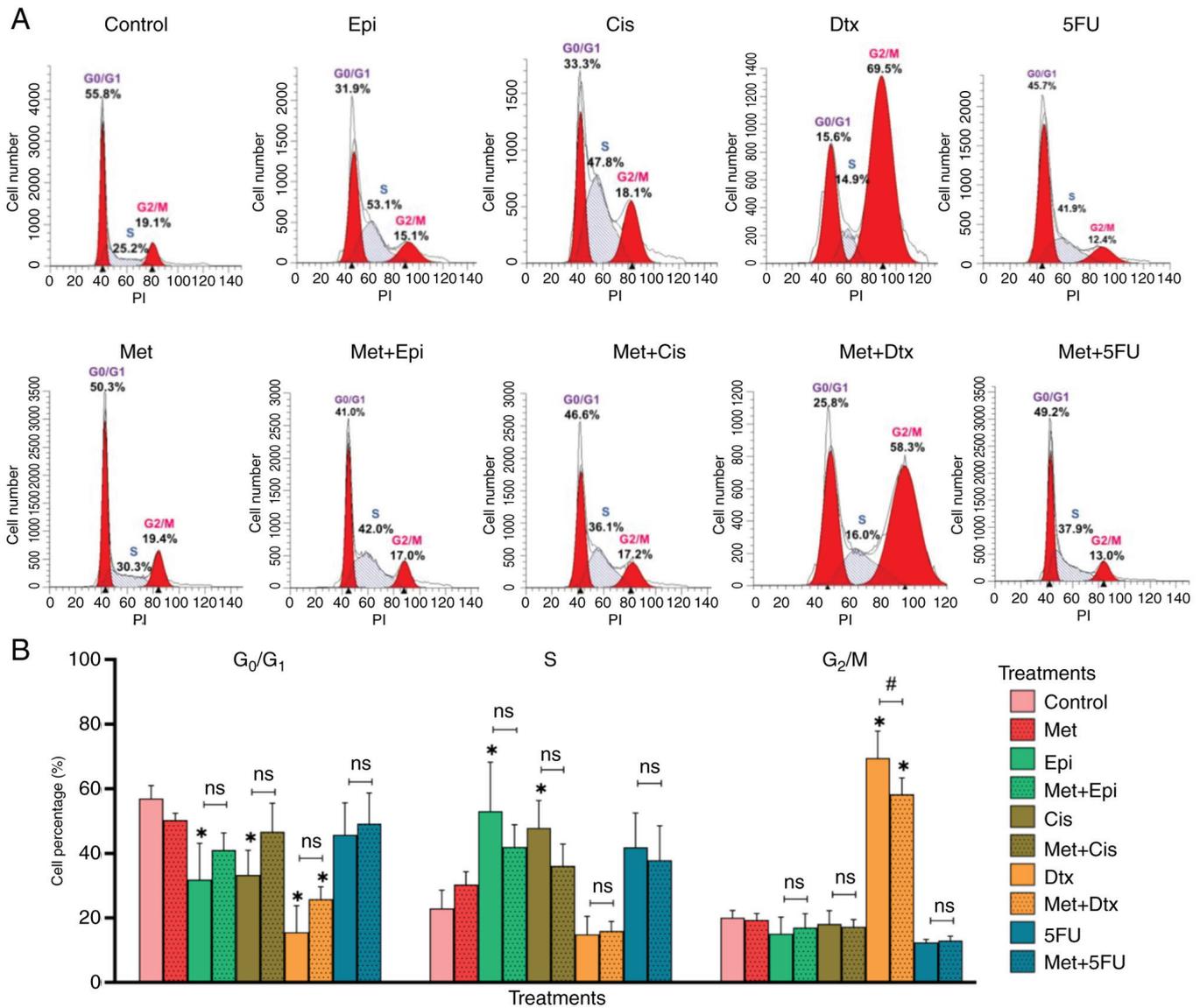
Figure 3. Effect of Met in combination with chemotherapy on cell cycle progression. (A) Flow cytometry plots of cells treated with Met alone or in combination with each chemotherapeutic drug. (B) Percentage of cells in the different phases of the cell cycle (G_0/G_1, S and G_2/M). Data are presented as the mean ± SD from three independent experiments performed in triplicate. *$P < 0.05$ vs. control; #$P < 0.05$. Met, metformin; Epi, epirubicin; Cis, cisplatin; Dtx, docetaxel; 5FU, 5-fluorouracil; ns, not significant.

with that in the control group ($P < 0.05$; Fig. 5A and B), as did the chemotherapy regimens ECF, DCF and CF ($P < 0.05$; Fig. 5C and D). Conversely, combining metformin with each chemotherapeutic drug or regimen significantly decreased cellular senescence ($P < 0.05$).

Metformin alone and in combination with chemotherapy decreases the clonogenic capacity of NCI-N87 GC cells. The present study demonstrated that metformin alone resulted in a significantly reduced number of GC cell colonies ($P < 0.05$; Fig. 6A and B). In addition, the four chemotherapy drugs had the same effect as metformin ($P < 0.05$). Notably, no significant differences were detected when metformin was combined with any of the chemotherapy drugs, in comparison to the chemotherapy drugs alone. No colonies were observed after GC cells were treated with each chemotherapy regimen alone or when combined with metformin (data not shown).

Discussion

Even though the incidence and mortality of GC have decreased in recent years (1), the prognosis of patients remains unfavorable due to chemoresistance. Developing new strategies to improve GC treatment is crucial, and metformin has attracted attention in the last few years as an antitumor agent (7). To the best of our knowledge, the present study was the first to demonstrate that metformin, in combination with chemotherapy regimens, induced cell death through increasing apoptosis accompanied by caspase activity and loss of ΔΨm. Furthermore, metformin significantly counteracted senescence induced by chemotherapy (Fig. 6C).

Apoptosis is considered the main mechanism of chemotherapy-induced cell death (25). This type of cell death is characterized by cell shrinkage, chromatin condensation,
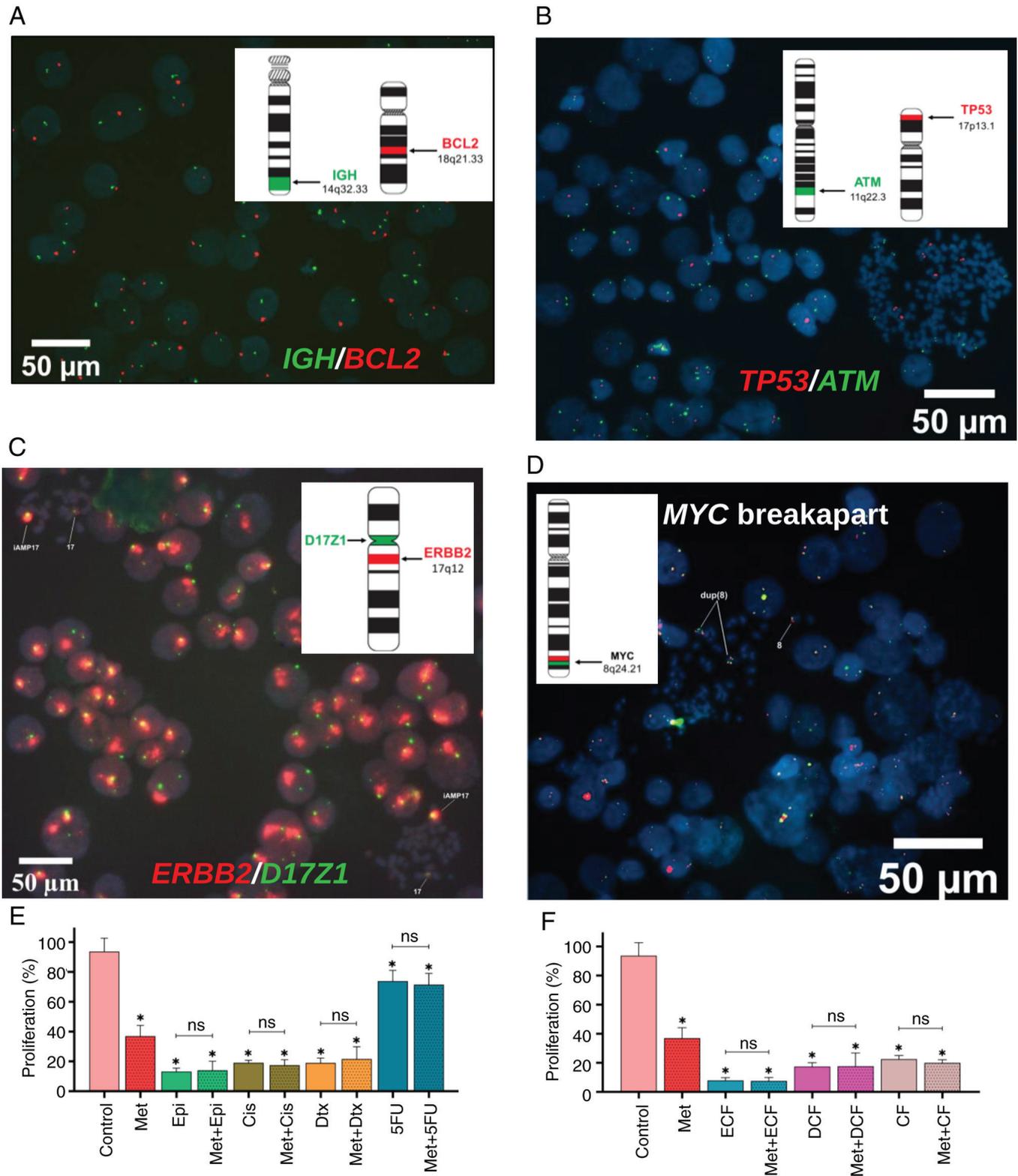
Figure 4. Effect of metformin in combination with chemotherapy on proliferation. (A) FISH with *IGH/BCL2* probe showed a deletion of the *BCL2* gene, (B) FISH with *TP53/ATM* disclosed a deletion of the *TP53* gene, (C) FISH with *ERBB2/D17Z1* probe showed an amplification of the *ERBB2* gene in all cells and (D) FISH with *MYC* breakapart probe revealed a gain of this gene. Proliferation of cells treated with metformin in combination with (E) each chemotherapeutic drug and (F) chemotherapy regimen. Data are presented as the mean ± SD from three independent experiments performed in triplicate. *$P<0.05$ vs. control. FISH, Fluorescence *in situ* hybridization; Met, metformin; Epi, epirubicin; Cis, cisplatin; Dtx, docetaxel; 5FU, 5-fluorouracil; ECF, epirubicin + cisplatin + 5-fluorouracil; DCF, docetaxel + cisplatin + 5-fluorouracil; CF, cisplatin + 5-fluorouracil; ns, not significant.

membrane budding, phosphatidylserine externalization and caspase activity (26). The present study revealed that NCI-N87 cells exhibited *TP53* deletion. Deletions and variants in this

gene have been reported in ~50% of different types of tumors, including breast, lung and ovarian cancer (27). In addition, deletion of the *BCL2* gene was detected in NCI-N87 cells,
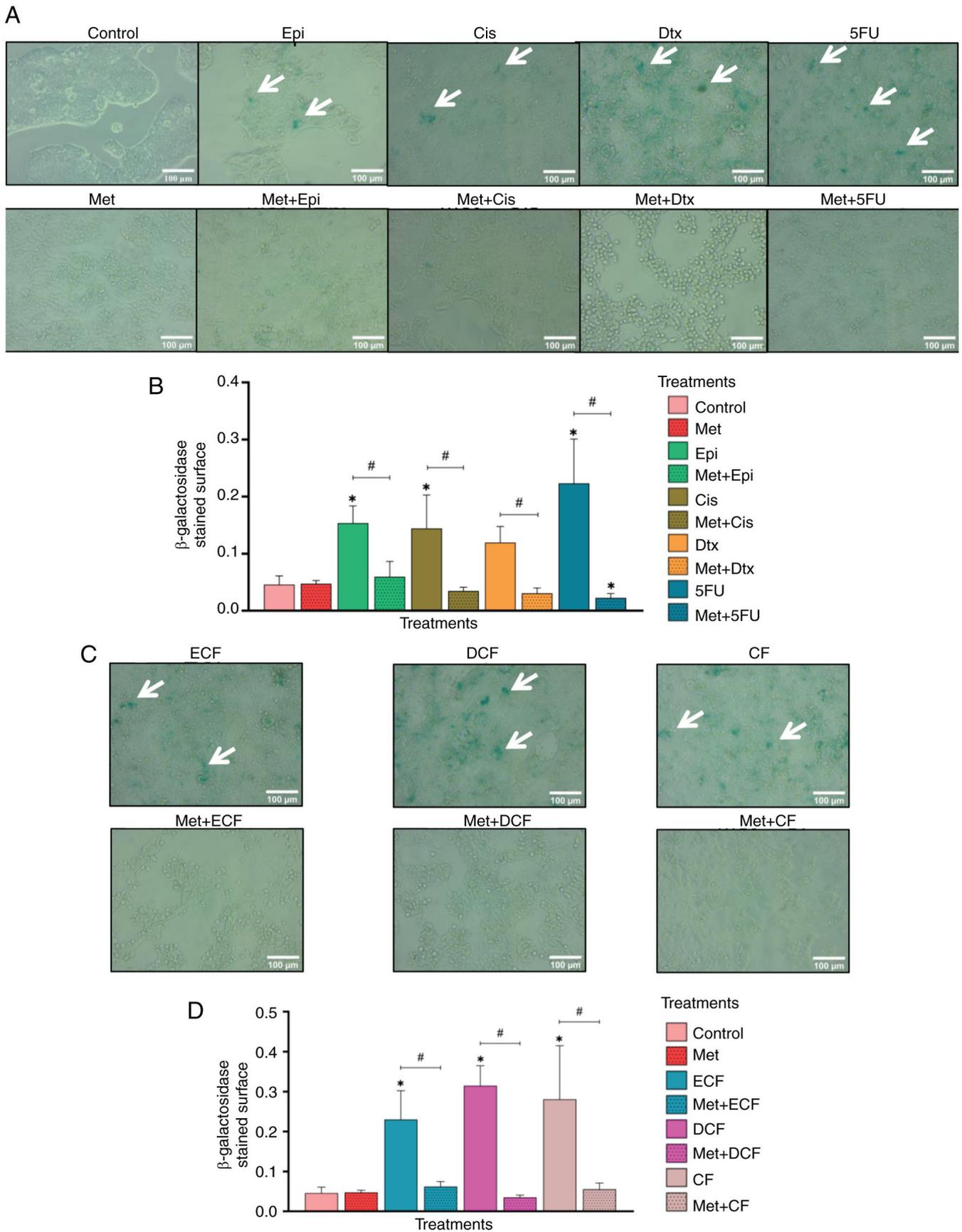
Figure 5. Effect of Met in combination with chemotherapy drugs on senescence. Cell senescence was assessed using β-galactosidase as a biomarker of senescence. Positive blue-stained cells were observed under a microscope at 40x magnification. (A) Representative images and (B) β-galactosidase stained surface of cells treated with Met in combination with each chemotherapeutic drug. (C) Representative images and (D) β-galactosidase stained surface of cells treated with Met in combination with each chemotherapy regimen. Data are presented as the mean ± SD from three independent experiments performed in triplicate. *P<0.05 vs. control; #P<0.05. Met, metformin; Epi, epirubicin; Cis, cisplatin; Dtx, docetaxel; 5FU, 5-fluorouracil; ECF, epirubicin + cisplatin + 5-fluorouracil; DCF, docetaxel + cisplatin + 5-fluorouracil; CF, cisplatin + 5-fluorouracil; ns, not significant.
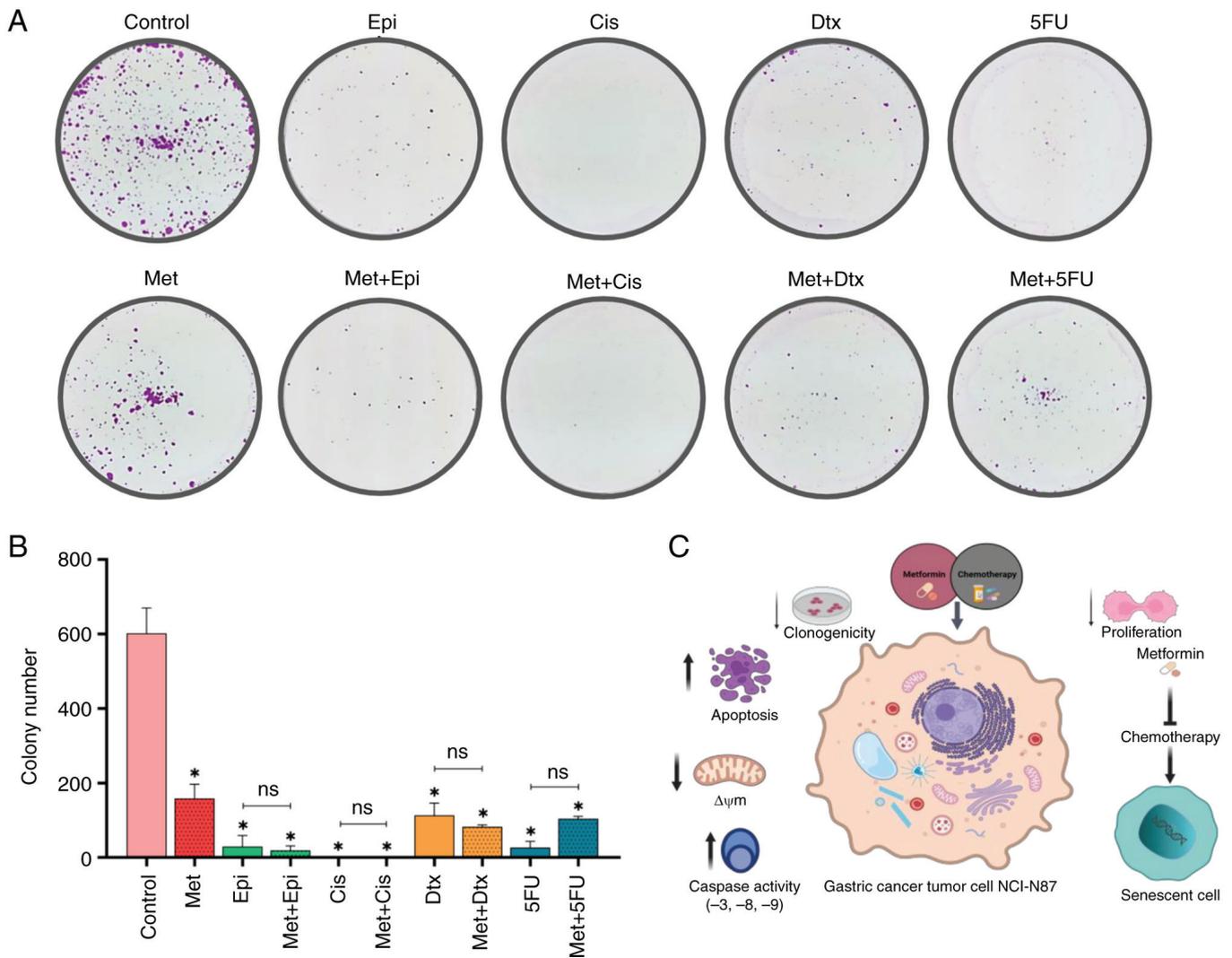
Figure 6. Effect of Met in combination with chemotherapy on clonogenic capacity. (A) Surviving cells after 15 days. (B) Number of colonies of surviving cells. (C) Integrative figure on the effect of metformin in combination with chemotherapy on NCI-N87 gastric cancer cells. Data are presented as the mean ± SD from three independent experiments performed in triplicate. *P<0.05 vs. control;. Met, metformin; Epi, epirubicin; Cis, cisplatin; Dtx, docetaxel; 5FU, 5-fluorouracil; ECF, epirubicin + cisplatin + 5-fluorouracil; DCF, docetaxel + cisplatin + 5-fluorouracil; CF, cisplatin + 5-fluorouracil; ns, not significant; ΔΨm, mitochondrial membrane potential.

which is interesting because this protein is anti-apoptotic and is generally overexpressed in tumor cells as a mechanism of cell death resistance (28). However, it is important to note that the present results indicated that *ERBB2* and *MYC* were amplified in NCI-N87 cells, suggesting that these cells have sustained proliferation, as has been observed in patients with GC (29).

The present results showed that metformin alone had a significant apoptotic effect on NCI-N87 GC cells. The same effect has been observed in previous studies; for example, treatment with metformin alone has been shown to significantly increase the apoptosis of GC cell lines (SGC7901 and BGC-823), and this effect was revealed to be correlated with inhibition of the HIF1α/PKM2 signaling pathway (15). The primary mechanism of action of metformin is through AMPK activation, resulting in mTOR inhibition. This effect has been demonstrated in three GC cell lines (MKN28, SGC-7901 and

BGC-823) and confirmed in a xenograft mouse model. In addition, metformin has been reported to decrease survivin expression (an anti-apoptotic protein) and increase apoptotic cells (18).

Additionally, AMPK activation and mTOR inhibition have been confirmed in AGS GC cells (16). Although the present study did not evaluate AMPK and mTOR, the present data are consistent with previous studies that support the idea that metformin induces apoptosis in GC models (15,16,18). Notably, one of the limitations of the present study is that the effects of metformin on non-tumor cells were not detected. However, it has been demonstrated in previous studies that metformin does not induce apoptosis in a human gastric epithelial cell line (GES-1) (18). Another study also demonstrated that metformin does not affect the viability of various non-tumor cells (normal human colon CCD 841 CoN cells, embryonic lung HEL 299 cells and 293 cells) (16).

The present study observed that metformin increased the apoptotic effect of 5-fluorouracil on NCI-N87 cells. A similar result was reported when metformin was combined with cisplatin, adriamycin or paclitaxel in AGS CG cells (19), and metformin combined with oxaliplatin in GC cells (SGC7901 and SNU-16) (20). Thus, this evidence suggests that metformin potentiates the apoptotic effect of some chemotherapy drugs in GC. Only one previous study combined metformin with more than one chemotherapy drug; metformin was shown to enhance tumor reduction when combined with cisplatin and rapamycin in mice, and this effect was revealed to be dependent on AMPK activation and mTOR inhibition (17). Notably, to the best of our knowledge, the present study is the first to assess the combination of metformin with chemotherapy regimens in an *in vitro* GC study model. The present results indicated that metformin can enhance the apoptotic effect of chemotherapy regimens ECF, DCF and CF, which are currently used for the treatment of patients with GC. These results may encourage future *in vivo* studies and clinical trials to determine the antitumor effect of metformin in the treatment of GC.

$\Delta\Psi$m is a reflection of mitochondrial functional status (30), and a higher $\Delta\Psi$m has been shown to be correlated with increased proliferation rate and tumorigenicity in 47DT human breast cancer cells (31). It has also been reported that cells with high $\Delta\Psi$m are resistant to apoptotic inducers (32). In the present study, it was demonstrated that metformin alone induced the loss of $\Delta\Psi$m in NCI-N87 GC cells. This effect was consistent with a previous report where metformin decreased $\Delta\Psi$m in AGS cells, and increased reactive oxygen species (ROS) levels and cytochrome *c* (16). These data indicated that loss of $\Delta\Psi$m is a key process in metformin-induced apoptosis. The four chemotherapy drugs that were used in the present study induced loss of $\Delta\Psi$m in NCI-N87 cells. Moreover, metformin in combination with chemotherapy drugs and DCF and CF regimens decreased $\Delta\Psi$m. Therefore, metformin in combination with chemotherapy could potentiate this effect, confirming the results observed in apoptosis.

Caspase activation indicates the beginning of apoptosis (26). It was hypothesized that metformin-induced apoptosis depends on caspase activity, as treatments with this drug resulted in a significant increase in caspase activity. Metformin alone significantly increased caspase activity, mainly that of caspases-3, -8 and -9. Similarly, in previous reports, metformin has been shown to increase caspase-3/7 activity in MKN-28, SGC-7901, BGC-823 and AGS GC cell lines (16,18). It has been proposed that metformin-induced apoptosis is via the intrinsic pathway, since increased caspase-9 activity has been shown to be correlated with loss of $\Delta\Psi$m, ROS levels and increased cytochrome *c* in AGS GC cells (16). However, the present findings suggested that metformin-induced apoptosis occurs by both extrinsic and intrinsic pathways since it activated both initiator caspases (caspase-8 and -9 for extrinsic and intrinsic pathways, respectively), which indicated that metformin-induced apoptosis could also be activated by the extrinsic pathway. This reinforces the apoptotic effect that metformin has on tumor cells. The fact that metformin can activate both apoptosis pathways is of importance since this mechanism would ensure that the cell will enter the apoptosis process through either of the two pathways, inhibiting resistance to cell death.

As aforementioned, only a few studies have investigated the effect of metformin in combination with chemotherapy. The present study observed a strong tendency of metformin to increase the caspase activity in combination with chemotherapy drugs and chemotherapy regimens. A recent study reported that metformin combined with oxaliplatin increased caspase-3 activity in SGC7901 and SNU-16 GC cell lines (20). Taken together, these findings indicated that in the process of apoptosis, metformin may help to enhance the apoptotic effect of both the individual drugs and the three chemotherapy regimens by increasing the loss of $\Delta\Psi$m, and the activation of initiator and effector caspases. However, it is essential to continue conducting studies to evaluate the participation of proapoptotic and anti-apoptotic proteins.

Tumor cells are characterized by uncontrolled proliferation due to the evasion of growth suppressors (33). The present study evaluated the progression of the cell cycle and proliferation. When cells were exposed to metformin, they accumulated in the G_0/G_1 phase, and proliferation was significantly decreased. These findings were similar to those reported in the MKN74 GC cell line, where most cells also accumulated in the G_0/G_1 phase. In addition, decreased expression of cyclin D1 and CDK4/6 was observed, as was decreased Rb phosphorylation; these are critical markers in the transition from G_0/G_1 to S phase (14). Other studies have also observed that GC cells accumulate in G_0/G_1 when exposed to metformin (15,17). Therefore, it may be inferred that metformin could induce cell cycle arrest in the G_0/G_1 phase in GC cells. To the best of our knowledge, the effect of metformin on cell cycle progression and proliferation in combination with chemotherapy in GC has not yet been evaluated. The present results showed that metformin does not modify the cell cycle phase in which the individual chemotherapy drugs act, nor were there any changes in the chemotherapy regimens; and no differences were detected regarding cell proliferation.

Cellular senescence is a state that can be triggered by stress or developmental signals, and is characterized by growth arrest, active metabolism, resistance to cell death and secretion of extracellular factors (34). The senescence-associated secretory phenotype (SASP) acts dually in cancer progression. On the one hand, it has an anti-neoplastic effect by recruiting immune system cells to premalignant lesions and promoting the repair of damaged tissues. By contrast, it has a pro-neoplastic effect that promotes proliferation, angiogenesis and inflammation due to the secretion of proinflammatory factors, such as IL-6, IL-8, MMPs and VEGF (35). Chemotherapy has been reported to induce the SASP, which has been shown to be correlated with chemoresistance; factors secreted by senescent cells can influence neighboring cells and promote tumor progression (36).

The present study investigated the effect of metformin and chemotherapy on senescence using the biomarker β-galactosidase. To the best of our knowledge, no studies have evaluated senescence in GC *in vitro* or *in vivo*, in response to a combination of metformin with conventional chemotherapy. No senescent cells were observed in response to treatment with metformin; however, as expected, chemotherapy induced senescence in NCI-N87 cells. A previous study suggested that the SASP could participate in chemoresistance, reducing therapy efficacy (37). Metformin could be proposed as an agent to suppress SASP as it is able to block the master transcription factor

NF-κB, which is required for the expression of numerous proinflammatory genes expressed in senescent cells (38). In addition, metformin decreased the mRNA expression of proinflammatory cytokines, such as CXCL5, IL-1B, IL-6 and IL-8 in fibroblasts and macrophages (38). Taken together, it may be suggested that metformin, besides not inducing senescence, can counteract the SASP induced by chemotherapy and consequently reduce one of the main obstacles in cancer treatment, chemoresistance.

Not all cells enter a cell death process after exposure to chemotherapeutic treatments; some become resistant or senescent. As aforementioned, surviving cells may contribute to tumor progression (39). Therefore, the present study evaluated the effect of the cells that survived after treatment. Although metformin is not an antitumor drug, it significantly decreased the clonogenic capacity of NCI-N87 GC cells. Similarly, this effect has been reported in N87 and MKN45 GC cells when exposed only to metformin (17). This indicates that metformin alone may decrease the clonogenic capacity of GC cells. The present study showed that metformin, when combined with 5-fluorouracil tended to increase the number of colonies, although this finding was not significant. A previous study reported that combining metformin with docetaxel or 5-fluorouracil can decrease the clonogenic capacity of AGS GC cells (21). These discrepancies could be due to the cell line evaluated. Although both cell lines are GC, each has its own genetic characteristics and, therefore, differences in gene expression. In addition, it is important to mention that in GC treatments, 5-fluorouracil is not administered as a monotherapy; it is combined with other antineoplastic drugs (40). Hence, it would be unlikely for the combination of metformin with 5-fluorouracil to be administered to patients as treatment for GC. Both the chemotherapy regimens and the combinations with metformin did not allow the formation of colonies of NCI-N87 GC cells. Therefore, these findings suggested that metformin does not interfere with the decrease in clonogenic capacity caused by chemotherapy.

The use of metformin in GC has not been sufficiently studied because clinical investigations are mainly observational studies in patients with DM2. There are studies that have evaluated the effect of metformin and the risk of developing GC, but the results are controversial. Different studies have detected no impact on the risk of developing GC of patients with DM2 when comparing those taking metformin with another type of treatment, such as sulfonylurea and insulin (41,42). However, other studies have reported that metformin reduces the risk of GC in patients who are prescribed metformin (43-45). These discrepancies may be related to biases and study design. In addition, previous studies have investigated the effect of metformin on survival; metformin has been reported to promote survival and decrease recurrence in patients with DM2 and GC after gastrectomy (46,47). It has also been reported that metformin improves overall survival in patients with DM2 and GC (48). However, another study reported that metformin had no impact on the survival of patients with GC and DM2 (49). Shuai *et al* (50) performed a systematic review and a meta-analysis to evaluate the effect of metformin on GC in patients with DM2 and revealed that the reduction in the incidence of GC was 21% (HR 0.790; 95% CI 0.624-1.001) (50). In order to provide more information about the effect of metformin on this type of cancer, prospective studies of patients with DM2 and clinical trials in patients with GC without DM2 are required.

The evidence from previous observational studies in patients indicates that metformin may reduce the risk of developing cancer (51), promote survival and could act as an adjuvant agent (52). In addition, *in vitro* and *in vivo* studies have reported that metformin alone, and in combination with chemotherapy may inhibit cellular growth and proliferation, suppress epithelial-mesenchymal transition, target stem cells, increase apoptosis and reduce tumor size (7,53).

Important considerations must be made in preclinical and clinical studies. The dose of metformin (10 mM) used in the present study was the median inhibitory concentration (IC_{50}), which is one of the parameters established *in vitro* studies (54,55). The IC_{50} is a measure commonly used in *in vitro* studies to evaluate the potency of a compound in inhibiting a certain biological response. It is also important to mention that the main objective of the present study was to investigate the molecular effects of the apoptosis process, and the desired effect was observed at this concentration. Currently, the doses of metformin used in a number of oncological trials have been shown to be effective for glucose control (56-58). Establishing the appropriate dose of metformin for use in cancer is necessary to safeguard patient safety and well-being.

In conclusion, the results of the present study indicated that metformin could be used as an adjuvant agent, since it could enhance the efficacy of chemotherapy regimens, increase apoptosis of tumor cells and counteract senescence induced by chemotherapy treatment, which may prevent or combat the chemoresistance that is associated with an unfavorable prognosis in this type of cancer.

Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

KCVI, JRCL, BGOT and LAPM performed the *in vitro* experiments. RMGA and JRGG performed the FISH experiments. KCVI, JYSL and PCOL analyzed data and wrote the manuscript. JYSL, TDPR and PCOL designed the study. JYSL and PCOL performed the final review and editing. PCOL supervised the study. PCOL, JYSL and KCVI confirm the authenticity of all raw data. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

This study was approved by the National Scientific Research Committee of Mexican Social Security Institute (approval number: R-2019-785-050; Guadalajara, Mexico).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Ferlay J, Colombet M, Soerjomataram I, Parkin DM, Piñeros M, Znaor A and Bray F: Cancer statistics for the year 2020: An overview. Int J Cancer: Apr 5, 2021 (Epub ahead of print).
2. Balakrishnan M, George R, Sharma A and Graham DY: Changing trends in stomach cancer throughout the world. Curr Gastroenterol Rep 19: 36, 2017.
3. Smyth EC, Verheij M, Allum W, Cunningham D, Cervantes A and Arnold D; ESMO Guidelines Committee: Gastric cancer: ESMO clinical practice guidelines for diagnosis, treatment and follow-up. Ann Oncol 27 (Suppl 5): v38-v49, 2016.
4. Chiurillo MA: Role of the Wnt/β-catenin pathway in gastric cancer: An in-depth literature review. World J Exp Med 5: 84-102, 2015.
5. Rawla P and Barsouk A: Epidemiology of gastric cancer: Global trends, risk factors and prevention. Prz Gastroenterol 14: 26-38, 2019.
6. Rena G, Hardie DG and Pearson ER: The mechanisms of action of metformin. Diabetologia 60: 1577-1585, 2017.
7. Courtois S, Lehours P and Bessède E: The therapeutic potential of metformin in gastric cancer. Gastric Cancer 22: 653-662, 2019.
8. Scarpello JHB and Howlett HCS: Metformin therapy and clinical uses. Diab Vasc Dis Res 5: 157-167, 2008.
9. Daugan M, Dufaÿ Wojcicki A, d'Hayer B and Boudy V: Metformin: An anti-diabetic drug to fight cancer. Pharmacol Res 113: 675-685, 2016.
10. Hua Y, Zheng Y, Yao Y, Jia R, Ge S and Zhuang A: Metformin and cancer hallmarks: Shedding new lights on therapeutic repurposing. J Transl Med 21: 403, 2023.
11. Graham GG, Punt J, Arora M, Day RO, Doogue MP, Duong JK, Furlong TJ, Greenfield JR, Greenup LC, Kirkpatrick CM, et al: Clinical pharmacokinetics of metformin. Clin Pharmacokinet 50: 81-98, 2011.
12. Morales DR and Morris AD: Metformin in cancer treatment and prevention. Annu Rev Med 66: 17-29, 2015.
13. Viollet B, Guigas B, Sanz Garcia N, Leclerc J, Foretz M and Andreelli F: Cellular and molecular mechanisms of metformin: An overview. Clin Sci (Lond) 122: 253-270, 2012.
14. Kato K, Gong J, Iwama H, Kitanaka A, Tani J, Miyoshi H, Nomura K, Mimura S, Kobayashi M, Aritomo Y, et al: The anti-diabetic drug metformin inhibits gastric cancer cell proliferation in vitro and in vivo. Mol Cancer Ther 11: 549-560, 2012.
15. Chen G, Feng W, Zhang S, Bian K, Yang Y, Fang C, Chen M, Yang J and Zou X: Metformin inhibits gastric cancer via the inhibition of HIF1α/PKM2 signaling. Am J Cancer Res 5: 1423-1434, 2015.
16. Lu CC, Chiang JH, Tsai FJ, Hsu YM, Juan YN, Yang JS and Chiu HY: Metformin triggers the intrinsic apoptotic response in human AGS gastric adenocarcinoma cells by activating AMPK and suppressing mTOR/AKT signaling. Int J Oncol 54: 1271-1281, 2019.
17. Yu G, Fang W, Xia T, Chen Y, Gao Y, Jiao X, Huang S, Wang J, Li Z and Xie K: Metformin potentiates rapamycin and cisplatin in gastric cancer in mice. Oncotarget 6: 12748-12762, 2015.
18. Han G, Gong H, Wang Y, Guo S and Liu K: AMPK/mTOR-mediated inhibition of survivin partly contributes to metformin-induced apoptosis in human gastric cancer cell. Cancer Biol Ther 16: 77-87, 2015.
19. Wu X: Effect of metformin combined with chemotherapeutic agents on gastric cancer cell line AGS. Pak J Pharm Sci 30: 1833-1836, 2017.
20. Zhu M, Wang J and Zhou R: Combination of metformin and oxaliplatin inhibits gastric cancer cell proliferation and induces apoptosis. Acta Biochim Pol 69: 321-326, 2022.
21. Fatehi-Agdam M, Vatankhah MA, Panahizadeh R, Jeddi F and Najafzadeh N: Efficacy of metformin and chemotherapeutic agents on the inhibition of colony formation and Shh/Gli1 pathway: Metformin/docetaxel versus metformin/5-fluorouracil. Drug Res (Stuttg) 71: 17-25, 2021.
22. Zarei E, Sefidi-Heris Y and Saadat I: Synergistic effects of metformin and curcumin on cytotoxicity of chemotherapy drugs using a gastric cancer cell line model. EXCLI J 20: 1488-1498, 2021.
23. Nomenclature ISCoHC: ISCN 2020: An International System for Human Cytogenomic Nomenclature. S. Karger AG, London, 2020.
24. Sun Y, Liu Y, Ma X and Hu H: The influence of cell cycle regulation on chemotherapy. Int J Mol Sci 22: 6923, 2021.
25. Ricci MS and Zong WX: Chemotherapeutic approaches for targeting cell death pathways. Oncologist 11: 342-357, 2006.
26. Elmore S: Apoptosis: A review of programmed cell death. Toxicol Pathol 35: 495-516, 2007.
27. Duffy MJ, Synnott NC, McGowan PM, Crown J, O'Connor D and Gallagher WM: p53 as a target for the treatment of cancer. Cancer Treat Rev 40: 1153-1160, 2014.
28. Strasser A and Vaux DL: Cell death in the origin and treatment of cancer. Mol Cell 78: 1045-1054, 2020.
29. Nevisi F, Yaghmaie M, Pashaiefar H, Alimoghaddam K, Iravani M, Javadi G and Ghavamzadeh A: Correlation of HER2, MDM2, c-MYC, c-MET, and TP53 copy number alterations in circulating tumor cells with tissue in gastric cancer patients: A pilot study. Iran Biomed J 24: 47-53, 2020.
30. Zhang BB, Wang DG, Guo FF and Xuan C: Mitochondrial membrane potential and reactive oxygen species in cancer stem cells. Fam Cancer 14: 19-23, 2015.
31. Yu M, Shi Y, Wei X, Yang Y, Zhou Y, Hao X, Zhang N and Niu R: Depletion of mitochondrial DNA by ethidium bromide treatment inhibits the proliferation and tumorigenesis of T47D human breast cancer cells. Toxicol Lett 170: 83-93, 2007.
32. Pietilä M, Lehtonen S, Närhi M, Hassinen IE, Leskelä HV, Aranko K, Nordström K, Vepsäläinen A and Lehenkari P: Mitochondrial function determines the viability and osteogenic potency of human mesenchymal stem cells. Tissue Eng Part C Methods 16: 435-445, 2010.
33. Hanahan D and Weinberg RA: The hallmarks of cancer. Cell 100: 57-70, 2000.
34. Lopes-Paciencia S, Saint-Germain E, Rowell MC, Ruiz AF, Kalegari P and Ferbeyre G: The senescence-associated secretory phenotype and its regulation. Cytokine 117: 15-22, 2019.
35. Campisi J: Aging, cellular senescence, and cancer. Annu Rev Physiol 75: 685-705, 2013.
36. Gilbert LA and Hemann MT: DNA damage-mediated induction of a chemoresistant niche. Cell 143: 355-366, 2010.
37. Chambers CR, Ritchie S, Pereira BA and Timpson P: Overcoming the senescence-associated secretory phenotype (SASP): A complex mechanism of resistance in the treatment of cancer. Mol Oncol 15: 3242-3255, 2021.
38. Moiseeva O, Deschênes-Simard X, St-Germain E, Igelmann S, Huot G, Cadar AE, Bourdeau V, Pollak MN and Ferbeyre G: Metformin inhibits the senescence-associated secretory phenotype by interfering with IKK/NF-κB activation. Aging Cell 12: 489-498, 2013.
39. Lee M and Lee JS: Exploiting tumor cell senescence in anti-cancer therapy. BMB Rep 47: 51-59, 2014.
40. Ajani JA, D'Amico TA, Bentrem DJ, Chao J, Cooke D, Corvera C, Das P, Enzinger PC, Enzler T, Fanta P, et al: Gastric cancer, version 2.2022, NCCN clinical practice guidelines in oncology. J Natl Compr Canc Netw 20: 167-192, 2022.
41. de Jong RG, Burden AM, de Kort S, van Herk-Sukel MP, Vissers PA, Janssen PK, Haak HR, Masclee AA, de Vries F and Janssen-Heijnen ML: No decreased risk of gastrointestinal cancers in users of metformin in the netherlands: A time-varying analysis of metformin exposure. Cancer Prev Res (Phila) 10: 290-297, 2017.
42. Zheng J, Xie SH, Santoni G and Lagergren J: Metformin use and risk of gastric adenocarcinoma in a Swedish population-based cohort study. Br J Cancer 121: 877-882, 2019.
43. Kim YI, Kim SY, Cho SJ, Park JH, Choi IJ, Lee YJ, Lee EK, Kook MC, Kim CG, Ryu KW and Kim YW: Long-term metformin use reduces gastric cancer risk in type 2 diabetics without insulin treatment: A nationwide cohort study. Aliment Pharmacol Ther 39: 854-863, 2014.
44. Tseng CH: Metformin reduces gastric cancer risk in patients with type 2 diabetes mellitus. Aging (Albany NY) 8: 1636-1649, 2016.
45. Dulskas A, Patasius A, Kaceniene A, Linkeviciute-Ulinskiene D, Zabuliene L and Smailyte G: A cohort study of antihyperglycemic medication exposure and gastric cancer risk. J Clin Med 9: 435, 2020.
46. Lee CK, Jung M, Jung I, Heo SJ, Jeong YH, An JY, Kim HI, Cheong JH, Hyung WJ, Noh SH, et al: Cumulative metformin use and its impact on survival in gastric cancer patients after gastrectomy. Ann Surg 263: 96-102, 2016.

47. Seo HS, Jung YJ, Kim JH, Lee HH and Park CH: The effect of metformin on prognosis in patients with locally advanced gastric cancer associated with type 2 diabetes mellitus. Am J Clin Oncol 42: 909-917, 2019.
48. Lacroix O, Couttenier A, Vaes E, Cardwell CR, De Schutter H and Robert A: Impact of metformin on gastric adenocarcinoma survival: A Belgian population based study. Cancer Epidemiol 53: 149-155, 2018.
49. Baglia ML, Cui Y, Zheng T, Yang G, Li H, You M, Xu L, Murff H, Gao YT, Zheng W, *et al*: Diabetes medication use in association with survival among patients of breast, colorectal, lung, or gastric cancer. Cancer Res Treat 51: 538-546, 2019.
50. Shuai Y, Li C and Zhou X: The effect of metformin on gastric cancer in patients with type 2 diabetes: A systematic review and meta-analysis. Clin Transl Oncol 22: 1580-1590, 2020.
51. Evans JMM, Donnelly LA, Emslie-Smith AM, Alessi DR and Morris AD: Metformin and reduced risk of cancer in diabetic patients. BMJ 330: 1304-1305, 2005.
52. Zhang J, Wen L, Zhou Q, He K and Teng L: Preventative and therapeutic effects of metformin in gastric cancer: A new contribution of an old friend. Cancer Manag Res 12: 8545-8554, 2020.
53. Cunha Júnior AD, Bragagnoli AC, Costa FO and Carvalheira JBC: Repurposing metformin for the treatment of gastrointestinal cancer. World J Gastroenterol 27: 1883-1904, 2021.
54. Sebaugh JL: Guidelines for accurate EC50/IC50 estimation. Pharm Stat 10: 128-134, 2011.
55. Brooks EA, Galarza S, Gencoglu MF, Cornelison RC, Munson JM and Peyton SR: Applicability of drug response metrics for cancer studies using biomaterials. Philos Trans R Soc Lond B Biol Sci 374: 20180226, 2019.
56. Drzewoski J and Hanefeld M: The current and potential therapeutic use of metformin-the good old drug. Pharmaceuticals (Basel) 14: 122, 2021.
57. Chae YK, Arya A, Malecek MK, Shin DS, Carneiro B, Chandra S, Kaplan J, Kalyan A, Altman JK, Platanias L and Giles F: Repurposing metformin for cancer treatment: Current clinical studies. Oncotarget 7: 40767-40780, 2016.
58. Saraei P, Asadi I, Kakar MA and Moradi-Kor N: The beneficial effects of metformin on cancer prevention and therapy: A comprehensive review of recent advances. Cancer Manag Res 11: 3295-3313, 2019.