

Combination effect of doxorubicin and HIF inhibitor on MCF-7 CD44⁺/CD24⁻ subpopulation cells in hypoxic condition

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Hypoxia-inducible factors (HIFs) and cancer stem cells (CSCs) are two challenging causes of radiotherapy and chemotherapy resistance, leading to most cases of failure and recurrence in breast cancer therapy. This study was conducted to investigate the inhibitory effect of combination therapy with doxorubicin (an anthracycline) and FM19G11 (an HIF inhibitor) on MCF-7 cells and their CSC-like cells (CSC-LCs). MCF-7 CSC-LCs with a CD44⁺/CD24⁻ phenotype were sorted and characterized by flow cytometry. A combination of doxorubicin and FM19G11 caused more cytotoxic effects on MCF-7 and CSC-LCs compared to doxorubicin monotherapy. The largest synergistic effect was observed in CSC-LCs under hypoxic conditions; however, MCF-7 cells showed no synergism in normoxic conditions. The administration of doxorubicin and FM19G11 induced late apoptotic and necrotic cell death in MCF-7 and CSC-LCs. Additionally, G2 phase arrest was observed in both cells. Our results demonstrated that co-administration of FM19G11 and doxorubicin had a synergistic effect in hypoxia and improved drug resistance in breast cancer stem cells.

Keywords: FM19G11. Combination Index. Hypoxia-induced factor. Cytotoxicity. Cancer stem cells.

INTRODUCTION

Breast cancer has been recognized as the most prevalent cancer and the primary cause of cancer-related death among females (Torre *et al.*, 2015). There are a variety of ameliorative therapies to improve the survival rate of breast cancer patients such as mastectomy, radiotherapy, hormone therapy, and chemotherapy (Theriault *et al.*, 2013). However, there is a pronounced therapeutic resistance to chemotherapy agents, endocrine therapy, and radiotherapy that has been attributed to the presence of breast cancer stem cells (BCSCs)

(Economopoulou, Kaklamani, Siziopikou, 2012). This phenomenon is mediated through multiple mechanisms such as overexpression of ABC transporters, particularly ABCG2 and P-glycoprotein efflux pumps, decreased reactive oxygen species, activation of DNA-damage checkpoints, and apoptosis resistance (Dean, Fojo, Bates, 2005; Phillips, McBride, Pajonk, 2006). CSCs, as a minute fraction of tumor cells, are characterized by self-renewal capability and anomalous activation of developmental signaling pathways. Genetic instability and epigenetic alterations contribute to the generation of CSCs from normal stem cells or differentiated cells, leading to hastened cancer establishment, metastasis, and recurrence. Thus, CSCs are prominent targets to surmount these obstacles (Nassar, Blanpain, 2016; Zhao *et al.*, 2018).

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Another factor that increases cancer therapeutic resistance is the accumulation of hypoxia-induced factors (HIFs), which were first identified by Semenza *et al.* in (1991). Indeed, HIFs as transcriptional factors engage in cell survival, proliferation, telomerase activation, genetic instability, angiogenesis, stem cell-related networks, glucose metabolism, invasion, metastasis and immune evasion (Burroughs *et al.*, 2013). Likewise, HIFs maintain self-renewal feature, a hallmark of CSCs, through modulation of pluripotency transcription factors (Oct-4, Nanog, Sox-2, and c-Myc) (Schöning, Monteiro, Gu, 2017). To date, different inhibitors have been developed to inhibit the HIF-1 α functions by blocking protein synthesis and stability, hetero-dimerization, HIF-1 α /DNA binding, and transactivation (Bhattarai, Xu, Lee, 2017), among which FM19G11 is a small molecule with low toxicity that inhibits HIF α protein at the mRNA level (Moreno-Manzano *et al.*, 2010). FM19G11 exerts its inhibitory effects via regulation of stemness maintenance genes, which is an ideal feature for spinal cord injuries and cancer therapy (Alastrue-Agudo *et al.*, 2018; Moreno-Manzano *et al.*, 2010). Moreover, it could efficiently increase endothelium-dependent vasodilatation via recruiting the PI3K/Akt/eNOS pathway (El Assar *et al.*, 2015).

The objective of this study is to investigate the suppressive effect of doxorubicin and FM19G11 on proliferation and cell cycle distribution of MCF-7 cells and their CD44⁺/CD24⁻ cancer stem cell-like cells (CSC-LCs) in normoxic and hypoxic conditions.

MATERIAL AND METHODS

Chemicals, reagents, and drugs

RPMI 1640 and Penicillin-Streptomycin Solution were obtained from Biosera (East Sussex, UK). MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide) was purchased from Carl Roth (Karlsruhe, Germany). Annexin V-FITC and propidium iodide (PI) were provided from Invitrogen (CA, USA) and Sigma-Aldrich (St. Louis, MO, USA), respectively. Doxorubicin was purchased from Pfizer (New York, USA). FM19G11 ([2-oxo-2-(p-tolyl) ethyl] 3-[(2, 4-dinitrobenzoyl) amino] benzoate, 3-[(2, 4-Dinitrobenzoyl) amino]-benzoic acid 2-(4-methylphenyl)-2-oxoethyl ester) was obtained from Sigma Aldrich.

Cell culture

MCF-7, a human breast adenocarcinoma cancer cell line, was purchased from Pasteur Institute Cell Bank of Iran (Tehran, Iran). CD44⁺/CD24⁻ cells (CSC-LCs) were sorted and characterized as previously reported (Sajadian *et al.*, 2015). The cells were maintained in 89% RPMI medium, 10% fetal bovine serum (FBS; Gibco, USA) and 1% Penicillin-Streptomycin at 37 °C and 5% CO₂. As for the hypoxic condition, the cells were grown in a hypoxia incubator chamber with 1% O₂ at 37 °C.

Viability assay

MTT assay was used to determine the cytotoxic effect of doxorubicin and FM19G11, alone and in combination, on the viability of CSC-LCs and MCF-7 cells. The cells were seeded at 1 \times 10⁴ per well in a 96-well plate and incubated at 37 °C overnight. Afterward, the cells were exposed to 0.625-20 μ M doxorubicin, 40-640 nM FM19G11 and their combination (doxorubicin with and without 40-640 nM FM19G11) for 24 h. Then, the MTT solution in PBS (5 mg/mL) was replaced with the medium and incubated for 4 h. Subsequently, 100 μ L dimethyl sulfoxide (DMSO) was added and absorbance was measured at 570 nm with a reference wavelength of 690 nm. The Combination Index (CI) was calculated using the Compusyn software (<http://www.combosyn.com/>) that measures the combination effect based on the Chou-Talalay method.

Apoptosis assay

For apoptosis assay, 5 \times 10⁵ cells/well were seeded into 6-well plates and incubated for 24 h. The defined concentrations of doxorubicin and FM 19G11 (concentrations that had a more synergistic effect, doxorubicin IC₅₀ in combination with 320 nM of FM19G11 for MCF-7 in hypoxic conditions, in combination with 320 nM of FM19G11 for CSC-LCs under normoxic conditions, and in combination with 40 nM of FM19G11 for CSC-LCs under hypoxic conditions) were added. Twenty-four hours after the treatment, the cells were harvested and re-suspended in 100 μ L binding buffer and stained with Annexin V (5 μ L) and PI (2 μ g/mL) for 15 min in the dark. The ratio of apoptotic and necrotic cells was quantified using flow cytometric analysis (BD Bioscience, San Jose, CA, USA).

Cell cycle assay

The cells were treated in the manner outlined for apoptosis assay for 24 h. The harvested cells were fixed in 70% cold ethanol for 2 h at 4 °C and incubated with 500 µL PI staining solution (Sajadian *et al.*, 2015) for 30 min at room temperature in the dark. Finally, analysis was done using the FlowJo software (Tree Star, Inc., Ashland, OR, USA).

Statistical analysis

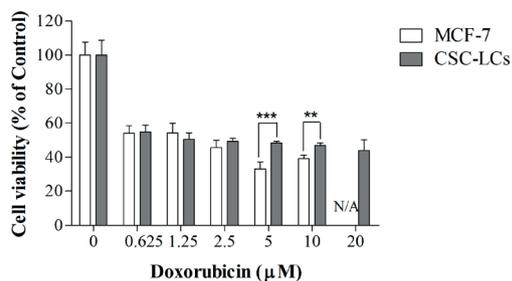
Statistical analysis was executed using the GraphPad Prism 5.01 (San Diego, CA). One-way analysis of variance followed by Turkey's post hoc test was applied for between-group comparison.

RESULTS

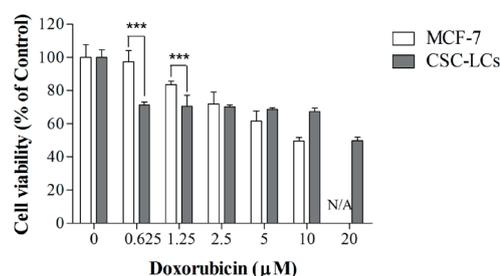
Cytotoxic effect of doxorubicin and FM19G11 on MCF-7 and CSC-LCs

MTT assay was used to evaluate the cytotoxic effect of doxorubicin with or without FM19G11 on MCF-7 and CSC-LCs in normoxic and hypoxic conditions. Exposure to doxorubicin and FM19G11 showed a tendency towards MCF-7 and CSC-LCs cell proliferation suppression in a dose-dependent manner in both hypoxic and normoxic conditions (Figure 1). Our results revealed that a hypoxic condition augmented drug resistance of MCF-7 and CSC-LCs (Table I). Next, the combination effect of doxorubicin and FM19G11 on MCF-7 and CSC-LCs was evaluated. According to Figure 2, co-treatment with the above drugs in some concentrations was associated with more cytotoxic effects compared to single-drug treated cells. Furthermore, in both cells, the combination of drugs was more efficacious in hypoxic versus normoxic conditions.

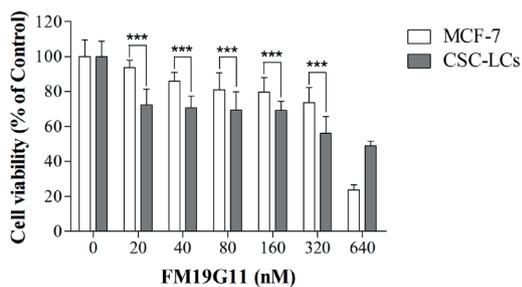
A



B



C



D

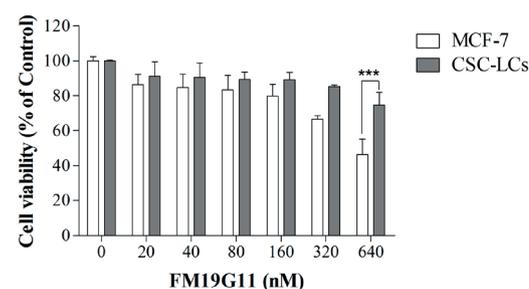


FIGURE 1 - Cytotoxic effect of doxorubicin and FM19G11 on MCF-7 and CSC-LCs in normoxic and hypoxic conditions. (A, C) Normoxia; (B, D) Hypoxia. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ as compared to corresponding concentration.

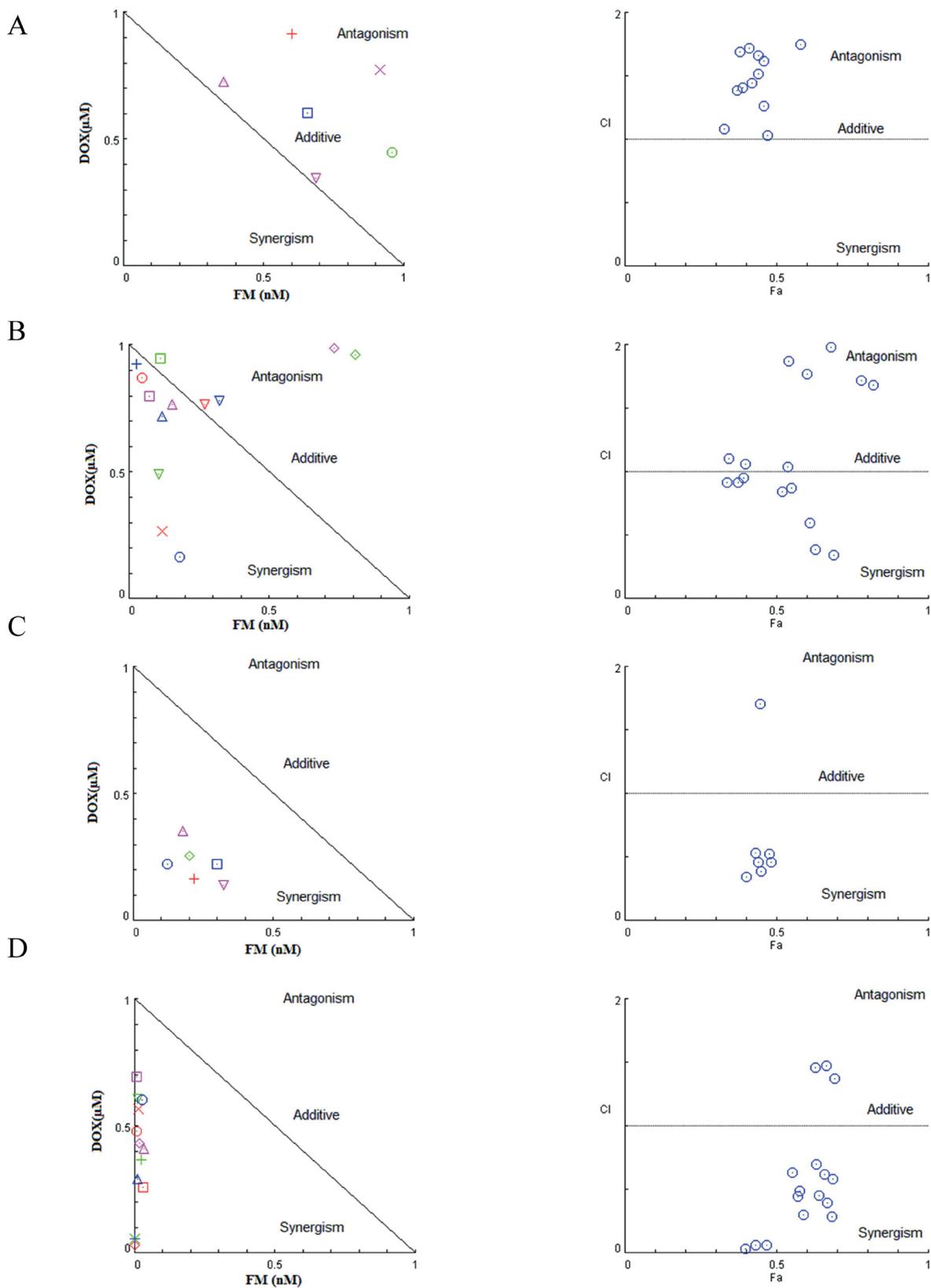


FIGURE 2 - Synergistic effect of doxorubicin (DOX) and FM19G11 (FM). (A) MCF-7 in normoxia; (B) MCF-7 in hypoxia; (C) CSC-LCs in normoxia; (D) CSC-LCs in hypoxia.

TABLE I - Cytotoxicity of the doxorubicin and FM19G11 against MCF-7 and CSC-LCs

	IC ₅₀		P value ^a
	MCF-7	CSC-LCs	
Doxorubicin (μM)	1.8 ± 0.03	2.3 ± 0.01	> 0.05
hypoxia	9.8 ± 0.05	19.5 ± 0.02	< 0.001
FM19G11 (nM)	400 ± 0.03	572 ± 0.02	< 0.05
hypoxia	560 ± 0.02	> 640	N/A

^a N/A: not available

Although administration of doxorubicin and FM19G11 caused no synergism under normoxic conditions in MCF-7 cells, additive and synergistic effects were observed in the hypoxic condition. In CSC-LCs, 320 nM FM19G11 in combination with any concentration of doxorubicin could induce synergism cytotoxicity under normoxic conditions. This effect was also achieved for 40 and 80 nM FM19G11, and all tested concentrations of doxorubicin under hypoxic conditions. Overall, better results were achieved for combination therapy (lower CI) in CSC-LCs relative to MCF-7 cells in similar conditions.

Effect of doxorubicin and FM19G11 on MCF-7 and CSC-LCs apoptosis

Annexin V and PI staining revealed that MCF-7 cells treated with doxorubicin at the IC₅₀ dose and 320 nM FM19G11 in hypoxic conditions underwent statistically significant late apoptotic and necrotic cell death (Figure 3). At this concentration, a marked late apoptosis (15.49% vs. 0.34%) and necrosis (13.5% vs. 0.94%) were detected in CSC-LCs compared to the control group under normoxic conditions. These percentages increased when CSC-LCs were exposed to doxorubicin IC₅₀ and 40 nM FM19G11 in hypoxic conditions compared to doxorubicin-treated cells. Our results indicated that the combination of doxorubicin and FM19G11 fortified necrotic cell death. However, no evident increase was found in programmed cell death in CSC-LCs upon treatment with doxorubicin, implying that these cells were resistant to doxorubicin-induced apoptosis.

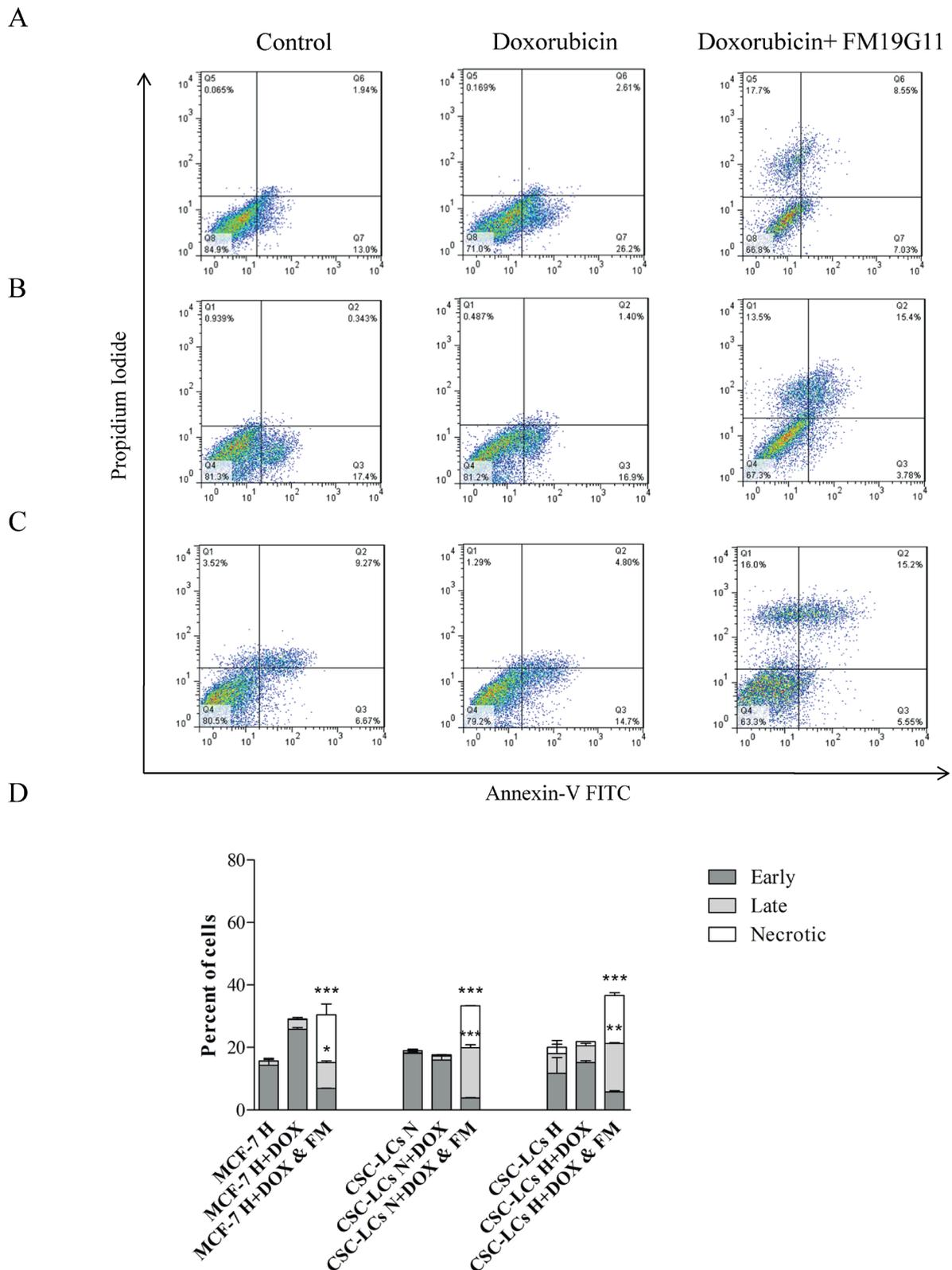


FIGURE 3 - Combination treatment with doxorubicin (DOX) and FM19G11 (FM) resulted in apoptotic and necrotic cell death in MCF-7 and their CSC-LCs in hypoxic (H) and normoxic (N) conditions. (A) MCF-7 cells in hypoxic condition; (B) CSC-LCs in normoxic condition; (C) CSC-LCs in hypoxic condition; (D) Percentage of cells that underwent apoptosis and necrosis. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ as compared to control group.

Effect of doxorubicin and FM19G11 on cell cycle distribution of MCF-7 and CSC-LCs

Cell cycle progression was quantitatively evaluated by flow cytometric analysis (Figure 4). MCF-7 and their CSC-LCs that received combination therapy in hypoxic and normoxic conditions respectively displayed a

weak accumulation in the G2/M phase. With regard to CD44⁺/CD24⁻ CSC-LCs in hypoxic conditions, a combination of doxorubicin and FM19G11 could arrest cells in the G2/M phase (25% vs. 15.04% of the control group; $P < 0.001$) with a modest reduction in the percentage of the S phase population (30.1% vs. 42.06% of the control group).

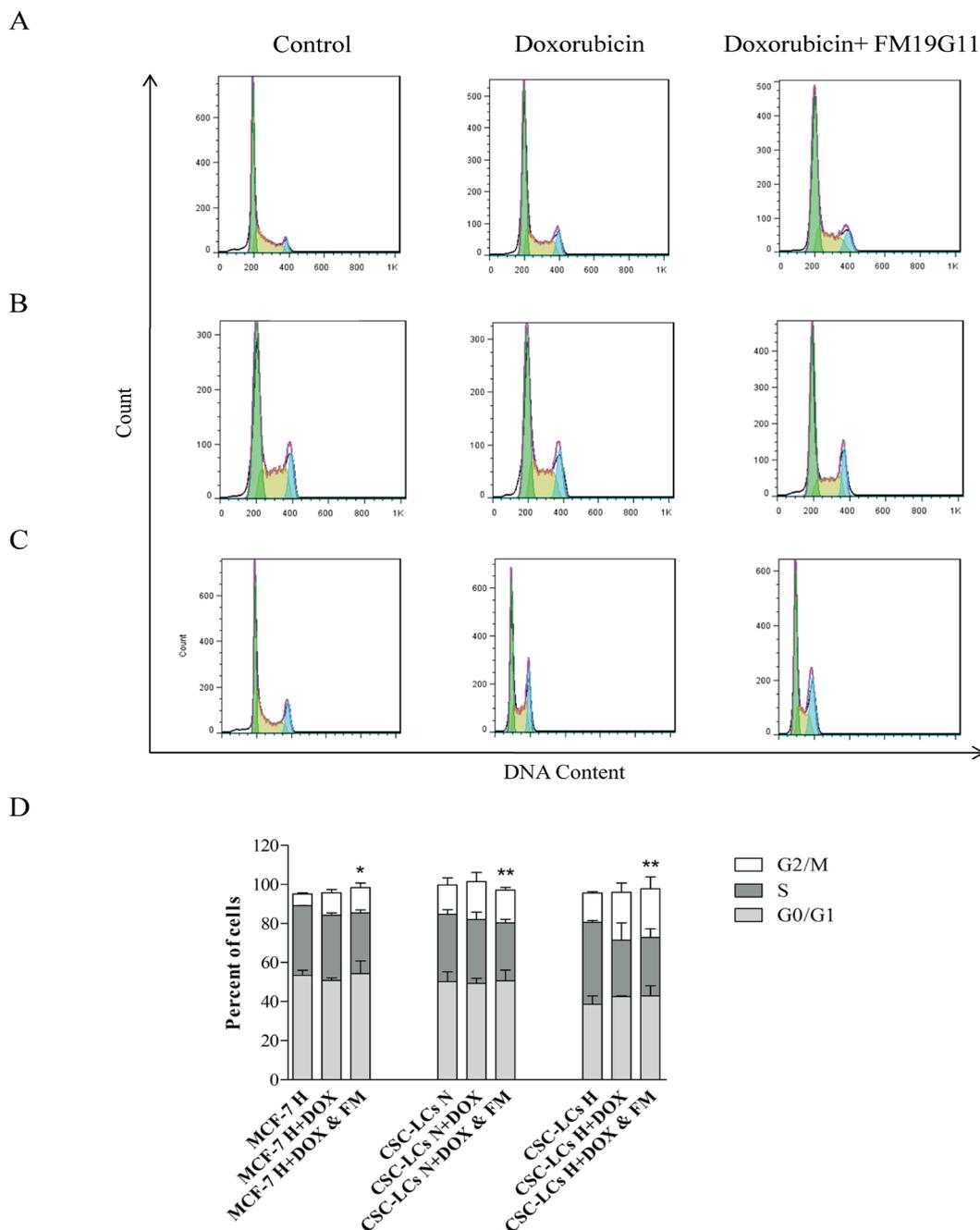


FIGURE 4 - Exposure to doxorubicin (DOX) and FM19G11 (FM) in hypoxic (H) and normoxic (N) conditions induced G2 phase cell cycle arrest. (A) MCF-7 cells in hypoxic condition; (B) CSC-LCs in normoxic condition; (C) CSC-LCs in hypoxic condition; (D) Cell cycle diagram. * $P < 0.05$, and ** $P < 0.01$ as compared to control group were referred to G2/M phase.

DISCUSSION

Breast cancer is known as a systematic disease with a stem cell origin. Since the first report of BCSCs by Al-Hajj *et al.*, (2003), several biomarkers have been proposed to identify them based on specific molecular markers using FACS analysis. The main markers of BCSCs are CD44⁺ and CD24^{-/low} (Dittmer, 2018). In fact, CD44⁺/CD24⁻ cells, which are most prevalent in basal-like/BRCA1 hereditary breast carcinoma with a triple negative state, have an essential role in proliferation, angiogenesis, and metastasis, causing poor prognosis (Giatromanolaki *et al.*, 2011). In addition, other markers such as ALDH1 (aldehyde dehydrogenase), ESA, CD133, CD61, and CD49f, as well as lack of expression of CD2, CD3, CD16, CD18, CD31, CD64, and CD140b are also used (Bozorgi, Khazaei, Khazaei, 2015; Velasco-Velázquez *et al.*, 2011). In this study, 37% of the CSC-LC population expressed CD44⁺/CD24⁻ biomarkers, which is consistent with a previous study (Villadsen *et al.*, 2007).

As mentioned earlier, up-regulation of ABC transporters hinders the intracellular retention of chemotherapeutic drugs such as doxorubicin in CSCs (Moitra, 2015). On the other hand, both HIF α subtypes are the main transcriptional regulators of ABC transporters. Besides, HIF1 α affects the CSC survival while HIF2 α contributes to stemness maintenance (Schöning *et al.*, 2017). Our results demonstrated that doxorubicin and FM19G11 could abolish cell proliferation in MCF-7 and CSC-LCs in a dose dependent manner. Meanwhile, higher IC₅₀ concentrations of the drugs which were observed in hypoxia and CSC-LCs, confirmed the induced therapeutic resistance. Doublier *et al.* (2012) also corroborated that HIF-1 α up-regulated P-gp expression accompanied by doxorubicin resistance in MCF-7 cells.

Combination therapy has been mostly used to overcome drug resistance and toxicity in multifactorial diseases like cancer or infectious diseases via affecting multiple signaling pathways (Bulusu *et al.*, 2016). Previous observations revealed the positive effect of combination therapy with chemotherapy drugs and HIF inhibitors. Samanta *et al.* (2014) reported that a combination of paclitaxel or gemcitabine with digoxin could ameliorate tumor control and prevent chemotherapy-induced breast cancer stem cells. Moreover, MSC (Se-methylselenocysteine), as an HIF-1 α inhibitor, in combination with irinotecan could sensitize human head and neck squamous cells to irinotecan in hypoxic conditions (Chintala *et al.*, 2010).

It should be noted that a combination of HIF inhibitors with other drugs could be used clinically, such as the use of temsirolimus (an mTOR/HIF inhibitor) combined with bevacizumab and liposomal doxorubicin in a phase I clinical trial (Moroney *et al.*, 2012).

In 1984, Paul Talalay presented the scientific term “CI: combination index” synergism (CI < 1), additive effect (CI = 1), and antagonism (CI > 1) (Chou, Talalay, 1984). Nowadays, several computational programs are available for CI calculation such as the Compusyn (Chou, 2006). Here, the MTT results of concurrent treatment with different concentrations of doxorubicin and FM19G11 justified the calculated CIs using the Compusyn. It was also demonstrated that treatment with FM19G11 increased the efficacy of doxorubicin against CSC-LCs under hypoxic conditions. The resulting data were in accordance with a recent report that a combination of FM19G11 and temozolamide (TMZ) in glioblastomas could reverse resistance in MGMT (O⁶-methylguanine DNA-methyltransferase) positive cells by fortifying the pro-apoptotic effect of TMZ (You *et al.*, 2018). In order to explore the growth inhibitory mechanism of doxorubicin and FM19G11, the treated cells were subjected to flow cytometric analysis. It was observed that co-administration of FM19G11 and doxorubicin resulted in 21% total apoptosis and 15% necrotic cells with G2/M arrest in CSC-LCs under hypoxic conditions. Previous investigations found that doxorubicin resulted in autophagy and necrosis by hyperactivation of PARP-1 (poly (ADP-ribose) polymerase-1) in response to DNA damage (Minotti *et al.*, 2004; Tacar, Sriamornsak, Dass, 2013). Nevertheless, Huang *et al.* (2012) claimed that autophagy is one of the reasons for doxorubicin resistance so that suppression of HMGB1 by shRNA in osteosarcoma decreases autophagy and increases sensitivity to doxorubicin.

CONCLUSION

A combination of doxorubicin and FM19G11 in MCF-7 and CD44⁺/CD24⁻ cells showed synergism in hypoxic conditions and increased the cytotoxic effects of doxorubicin. This combination resulted in a G2/M arrest in the hypoxic condition in both cells and fortified apoptosis in MCF-7 and CD44⁺/CD24⁻ CSC-LCs in both conditions. The results disclosed here demonstrated that HIF inhibitors in combination with cytotoxic agents could decrease the drug resistance and increase the therapeutic response.

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