Dose-reducing synergistic effects of doxorubicin and 5-fluorouracil on triple-negative breast cancer cells

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Abstract. Triple-negative breast cancer (TNBC) is an aggressive subtype of cancer with limited treatment options. Despite the use of both doxorubicin (Doxo) and 5-fluorouracil (5-FU) as part of the FAC regimen, their interaction in TNBC has not yet been studied in detail, at least to the best of our knowledge. As reducing toxicity while preserving therapeutic efficacy remains a key therapeutic goal, the present *in vitro* study investigated whether the combination of Doxo and 5-FU exerts synergistic effects on MDA-MB231 cells and enables dose reduction. MTT assays were used to measure the viability of the TNBC cell line, MDA-MB-231 and non-transformed rat embryo fibroblasts (REFs) following exposure to Doxo and 5-FU, alone or in combination, for 72 h. Synergistic effects were evaluated using the combination index (CI) calculated using CalcuSyn software. Cell morphology was assessed by inverted microscopy, and apoptosis was assessed using acridine orange/ethidium bromide dual staining followed by fluorescence microscopy. The dose-response data revealed anti-proliferative effects in MDA-MB-231 cells (GI_{50}=8.57 and 34.38 μM for Doxo and 5-FU, respectively) and REF cells (GI_{50}=1.3 and 48.66 μM, respectively). Combination treatment reduced the concentrations of Doxo and 5-FU required to achieve the GI_{50} by 27.9 and 85.5%, respectively, compared with monotherapy. This dose-sparing effect was accompanied by a modest increase in apoptosis and marked morphological alterations. To the best of our knowledge, the present study is the first to quantitatively characterize the synergistic interaction between Doxo and 5-FU in TNBC cells, providing evidence of clinically relevant dose reduction beyond the historical empirical use of the FAC regimen.

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Introduction

Triple-negative breast cancer (TNBC) is the most aggressive subtype of breast cancer affecting women, accounting for 10-20% of all breast cancer diagnoses (1). It is characterized by the absence of three major cellular receptors [estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2) protein]. These receptors are exploited as druggable targets in the majority of breast cancer treatments, including hormone therapy and HER2-targeted therapy (2). As a result, treatment options for TNBC are more limited than those for other breast cancer subtypes, which may contribute to the higher rates of treatment failure and disease recurrence observed in these patients (3).

TNBC is associated with a particularly high risk of recurrence as it does not respond to hormone therapy or HER2-targeted treatments. Additionally, it has the potential to metastasize beyond the breast to colonize other parts of the body due to the development of drug resistance to conventional cancer therapeutic regimens. Thus, patients with TNBC may require stronger therapeutic protocols, such as chemotherapy, which can increase treatment-associated side-effects (4). Since 2018, five novel therapies have been implemented in clinical practice for the management of advanced TNBC. Poly(ADP-ribose) polymerase inhibitors constitute the only genomically targeted treatment currently available; however, they are applicable only to the small subset of patients carrying germline pathogenic mutations in BRCA1 or BRCA2. Pembrolizumab remains the only PD-1 checkpoint inhibitor sanctioned in the USA for first-line use in conjunction with chemotherapy, representing a treatment option for ~40% of patients with PD-L1-positive malignancies. Antibody-drug conjugates have also markedly advanced the treatment of advanced-stage TNBC. However, these agents are not TNBC-specific, as both sacituzumab govitecan and trastuzumab deruxtecan have demonstrated efficacy in other breast cancer subtypes (5). By contrast, the 5-fluorouracil (5-FU), doxorubicin (Doxo) and cyclophosphamide (FAC) regimen is currently largely restricted to adjuvant or neoadjuvant-stage breast cancer and is not routinely used in relapsed/metastatic TNBC protocols. This highlights the need to re-evaluate and optimize established chemotherapeutic regimens by simplifying their composition and exploring synergistic dose-reduction strategies (6).

5-FU is an antimetabolite chemotherapeutic agent that is extensively used in the treatment of cancer, particularly colorectal cancer. It exerts its anticancer effects by inhibiting thymidylate synthase (TS) and incorporating its metabolites into RNA and DNA, thereby disrupting nucleic acid synthesis and impairing cellular replication (7). Doxo is an anthracycline antibiotic employed in the treatment of several malignancies, including lung, thyroid, non-Hodgkin's and Hodgkin's lymphoma, gastric and breast cancer. It functions primarily by inhibiting topoisomerase II, an enzyme essential for DNA replication and repair, thereby disrupting DNA and RNA synthesis and ultimately inducing cell death (8). In TNBC, 5-FU continues to be used in combination with anthracyclines as part of established treatment protocols, reflecting the limited availability of targeted therapies (9,10). Recently, an MDA-MB231 xenograft study reported that the combination of Doxo + 5-FU resulted in more extensive metabolic pathway disruption and greater antitumor effects than either drug alone, providing mechanistic support for combination therapy in TNBC (11). Given the high rates of treatment failure and disease recurrence associated with TNBC, repurposing combinations of established, FDA-approved chemotherapeutic agents represents a promising therapeutic strategy.

Despite the availability of clinical data, *in vitro* research using MDA-MB-231 cells that quantifies drug-drug synergy through combination index analysis, while linking dose-sparing to apoptosis and morphology remains limited. To address this gap, the present study revisited this drug combination to determine whether synergistic interactions could enable dose reduction. Accordingly, the present study investigated the combined effects of these agents in the TNBC cell line, MDA-MB-231, using apoptosis and morphological changes as the primary outcome measures.

Materials and methods

Cells, cell culture and drug preparation. The TNBC cell line, MDA-MB-231 (ATCC HTB-26), which lacks the expression of the ER, PR and HER2 proteins, was used in the present study as an *in vitro* model of TNBC (12). The MDA-MB-231 cells were kindly supplied by the Faculty of Science, Baghdad University, Baghdad, Iraq. Furthermore, non-transformed rat embryo fibroblasts (REFs) served as a model for normal cells (13). The REF cell line was kindly provided by the Biotechnology Research Center at Al-Nahrain University, Baghdad, Iraq. It was originally established by the Experimental Therapy Department at the Iraqi Center for Cancer and Medical Genetic Research (ICCMGR), Mustansiriyah University, Baghdad, Iraq. Both cell lines were grown in RPMI-1640 medium (Capricorn Scientific) enriched with 10% heat-inactivated fetal bovine serum (FBS) and 1% penicillin-streptomycin (Euroclone S.p.A.) in a humidified incubator (Sanyo; PHC Corporation) at 37˚C with 5% CO_2 in tissue culture flasks (14). Additionally, stock solutions of both Doxo (cat. no. ab120629) and 5-FU (cat. no. ab142387), provided by Abcam, were dissolved in DMSO (United States Biological Life Sciences). The working solution of the aforementioned drug was prepared at a concentration of 10 mM and subsequently diluted with complete medium, before being stored in the dark and frozen at -20˚C.

Assessment of cultured cell viability by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cytotoxic effects were evaluated using an *in vitro* MTT assay. A total of 7,000 cells were inoculated in each well of a 96-well plate and incubated overnight at 37˚C with 5% CO_2 to facilitate cell adherence. The cells were then treated with increasing concentrations of 5-FU (0.01-50 μM) or Doxo (0.01-40 μM), as specified. Each treatment used three technical replicate wells per experiment. Single-agent dose-response experiments in MDA-MB-231 cells (Fig. 1A and B) were performed across three independent biological replicates (n=3), each with three technical triplicate wells. Single-agent dose-response experiments in REFs (Fig. 1C and D) were performed in a single biological replicate (n=1) with three technical triplicate wells, representing a limitation of the present dataset (as explained below in the Discussion). Following incubation, the medium was removed from the plate, and 20 μl MTT solution (5 mg/ml; Shanghai Macklin Biochemical Co., Ltd.) were added to each well and incubated for 3 h at 37˚C in the absence of light, as previously described (15). To dissolve the MTT, 50 μl DMSO were added, followed by 10 min of agitation. A microplate reader (BioTek; Agilent Technologies, Inc.) was then used to assess the absorbance at 490 nm. Cell viability was calculated using the following equation: Viability%=A(treated)/A(untreated) x100, where 'A' denotes the absorbance. Dose-response curves were generated using GraphPad Prism software (version 10.0.0; Dotmatics), and the concentration required to reduce cell viability by 50% (GI_{50}) was determined from the fitted curves, as previously described (16).

Assessment of the synergistic effects of 5-FU and Doxo on MDA-MB-231 cells. The synergistic effects of 5-FU and Doxo on the MDA-MB-231 cells was evaluated using an MTT assay following treatment with various concentrations of the tested drugs on the basis of the determined GI_{50} (0.25x, 0.5x, 1x, 2x and 4x for each drug), as previously described (17). Chou-Talalay combination index (CI) CalcuSyn software (version 2.1; Biosoft) was used to evaluate the synergistic effect of the combined effects of both drugs (5-FU and Doxo) (18). CI values of 0.1-0.9, 0.9-1.1 and >1.1 denote synergistic, additive and antagonistic interactions, respectively (18).

Determination of the dose required to achieve the desired GI_{50}. The extent of dose-sparing achieved by combining Doxo and 5-FU was determined using CalcuSyn software (version 2.1; Biosoft) based on the median-effect principle of Chou (18). For each drug, the dose required to achieve 50% growth inhibition (GI_{50}) in combination was expressed as a fraction of the dose required to achieve the same effect when the drug was used alone, with the single-agent dose derived from the corresponding single-agent dose-response curve generated within the same experimental replicate: Fold of dose required=dose in combination/dose alone. A value of <1 indicates that a lower dose of the combination was needed to achieve the same effect as the single agent. This value was calculated for both Doxo and 5-FU across two independent biological replicates and is reported as the mean ± SEM.

Assessment of the effects of 5-FU and Doxo treatment on the morphology of MDA-MB-231 cells. The effects of

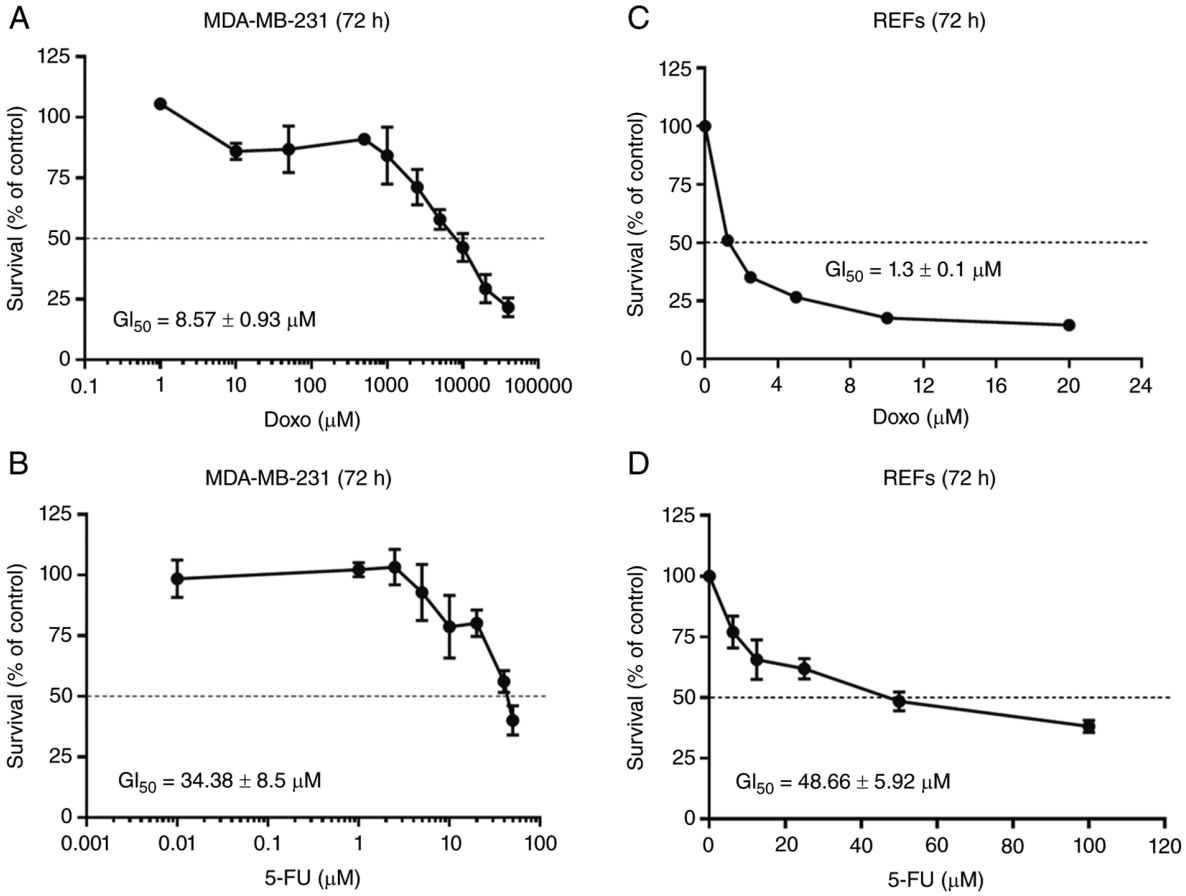

Figure 1. Cytotoxic effects of Doxo and 5-FU as single agents on MDA-MB-231 cells and REFs. MTT assays were used to assess cell viability after the (A and B) MDA-MB-231 cells, and (C and D) REFs were exposed to increasing concentrations of (A and C) Doxo or (B and D) 5-FU for 72 h. Data points are displayed as the mean percentage survival ± SEM. (A and B) GI_{50} values for MDA-MB-231 cells were determined from three independent biological replicates; (C and D) GI_{50} values for REF cells were determined from a single biological replicate with technical triplicate measurements. Doxo, doxorubicin; 5-FU, 5-fluorouracil; REFs, rat embryo fibroblasts.

5-FU and Doxo treatment, both alone and in combination, on the MDA-MB-231 cells were investigated by seeding the MDA-MB-231 cells at a density of $25x10^3$ cells on a flat-bottom 24-well tissue culture plate. After 24 h, the cultured MDA-MB-231 cells were exposed to 5-FU (10 μM) or Doxo (10 μM) individually or in combination and incubated at 37˚C, 5% CO_2 and 95% humidity for 72 h. The cell shape was then examined at x200 magnification using an inverted microscope (Meiji Techno) equipped with a digital camera, as previously described (13).

Assessment of apoptosis induction by fluorescence microscopy. Apoptosis was assessed by acridine orange (AO)/ethidium bromide (EB) dual fluorescence staining. AO stains the nuclei of viable and apoptotic cells green following DNA binding, whereas EB only penetrates cells with compromised plasma membranes, staining dead cells red. The differential staining pattern enables discrimination between viable, apoptotic and necrotic cells. In brief, the MDA-MB-231 cells were cultured at a density of $21x10^3$ cells/well on a 24-well flat-bottomed tissue culture plate. The MDA-MB-231 cells were then exposed to 5-FU and Doxo, both individually and in combination, for an incubation period of 72 h at 37˚C with 5% CO_2. Subsequently, the cultured cells were rinsed with PBS, trypsinized, detached and harvested in 1.5-ml tubes. The cultured cells were then stained with 1 μl of double-staining solution composed of 100 μg of each AO or EB (Fluka Chemie GmbH) to obtain 9 μl of cell suspension. A drop of the gently mixed combination (cells + AO/EB dye) was then placed on a clean slide and covered with a coverslip to be examined by a fluorescence microscope (HumaScope, HUMAN Diagnostics Worldwide), as previously described (19). A total of 100 cells were manually counted by visual inspection under the fluorescence microscope and spotted within 20 min to stratify their morphology into different stages of apoptosis. Viable cells exhibited bright green nuclei with an intact nuclear morphology. By contrast, early apoptotic cells displayed green cytoplasm with condensed yellow chromatin, whereas late apoptotic cells exhibited orange nuclei with segmented chromatin. Necrotic cells were identified by red nuclei with uniformly condensed chromatin (20).

Statistical analysis. The raw data used in the present study were statistically analyzed using GraphPad Prism software (version 10.0.0; Dotmatics) and Microsoft Excel 2024 (Microsoft Corporation). Combination index (CI) values at ED50, ED75 and ED90 (Fig. 2B) were compared using one-way ANOVA followed by Tukey's multiple-comparisons test. Fold-reduction values for each drug (Fig. 2D) were compared with a theoretical value of 1, representing no dose-sparing

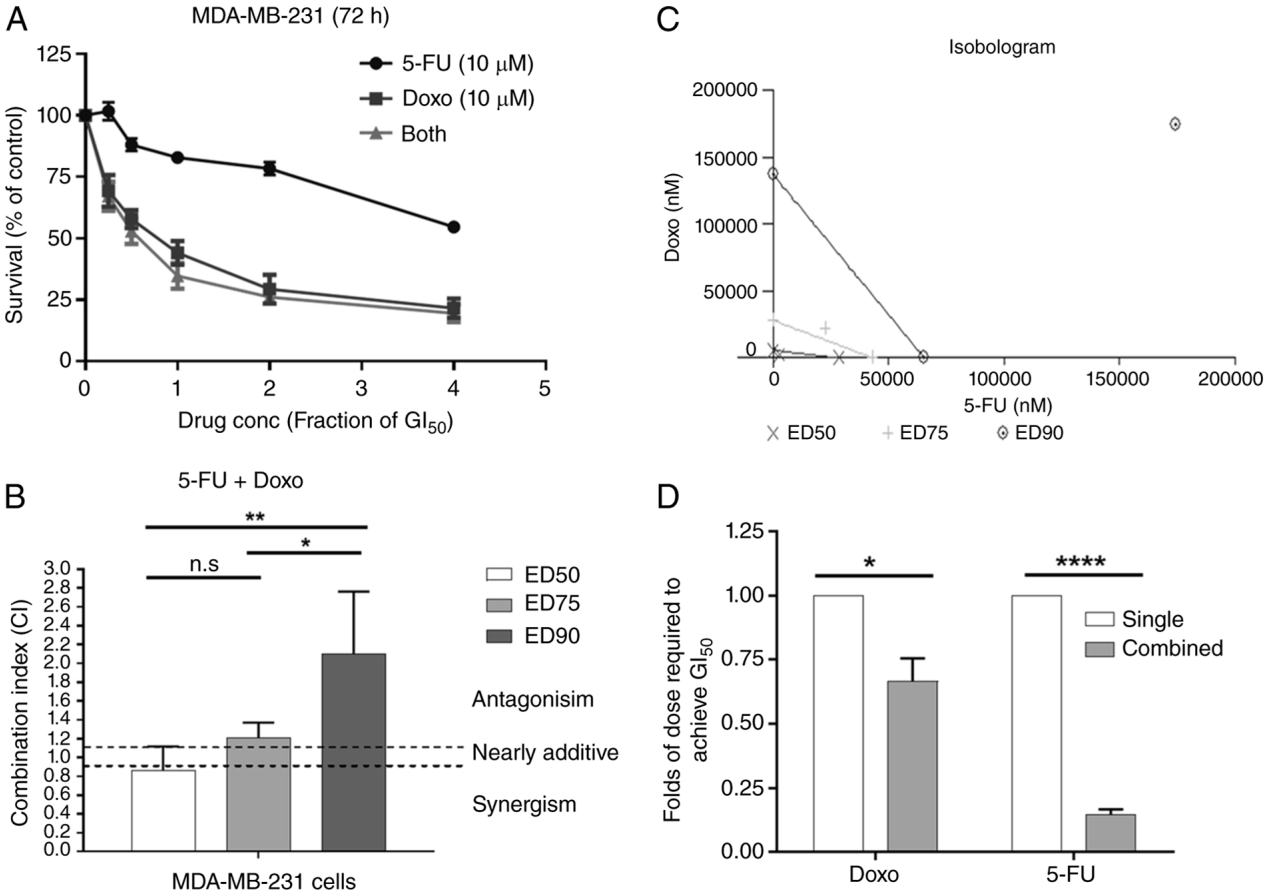

Figure 2. Synergistic cytotoxic effects of Doxo and 5-FU in MDA-MB-231 cells. (A) Dose-response curves of MDA-MB-231 cells incubated for 72 h with fractions of the GI_{50} of 5-FU alone, Doxo alone, or a combination of both, prior to the assessment of cell viability by MTT assay. (B) Mean CI values computed using CalcuSyn software at the ED50, ED75 and ED90 (one-way ANOVA, P=0.0020; Tukey's multiple comparisons test): n.s, not significant (ED50 vs. ED75); **P<0.01 (ED50 vs. ED90); *P<0.05 (ED75 vs. ED90). (C) Isobologram illustrating the Doxo/5-FU drug interaction points at different effective doses in relation to their respective additivity lines. Points falling below the line indicate synergism, whereas those approaching or above the line reflect additive or antagonistic effects. (D) Fold reduction in the doses of Doxo and 5-FU required to achieve the GI_{50} with combination therapy compared with monotherapy. *P<0.05 and ****P<0.0001 vs. a theoretical value of 1 (one-sample t-test performed separately for Doxo and 5-FU). Data points on the (A) survival curves and (B and D) bars were generated based on the average of two biological replicates with triplicate measurements, expressed as the mean ± SEM. Doxo, doxorubicin; 5-FU, 5-fluorouracil; conc, concentration.

effect, using a one-sample t-test performed separately for Doxo and 5-FU. Data are presented as the mean ± SEM, and a threshold value of P<0.05 was considered to indicate a statistically significant difference. All experiments were performed using 2-3 biological replicates; however, only a single biological replicate was available (REF single-agent dose-response, Fig. 1C and D; apoptosis quantification, Fig. 3C).

Results

Effects of 5-FU and Doxo as single agents on the viability of MDA-MB-231 cells and REFs. The effects of 5-FU and Doxo as single agents on the viability of aggressive TNBC cells (MDA-MB-231) cells were assessed using the MTT colorimetric assay. Following 72 h of exposure to each agent, cell viability decreased in a concentration-dependent manner, although the cells displayed different sensitivities to each drug (Fig. 1). Compared with the MDA-MB-231 cells treated with 5-FU, the MDA-MB-231 cells treated with Doxo exhibited a greater sensitivity to treatment (mean GI_{50}=8.57±0.93 μM; Fig. 1A) (mean GI_{50}=34.38±8.5 μM; Fig. 1B).

To assess the selectivity of each drug for cancer cells vs. normal cells, GI_{50} values were also determined in REFs, the only non-transformed cell line available in the Biotechnology Research Center, Al-Nahrain University. Compared with the MDA-MB-231 cells, the REFs were considerably more sensitive to Doxo (GI_{50}=1.3±0.1 μM; Fig. 1C). By contrast, the REFs were less sensitive to 5-FU than the MDA-MB-231 cells (GI_{50}=48.66±5.92 μM; Fig. 1D).

Co-treatment of the MDA-MB-231 cells with the 5-FU/Doxo combination exerts synergistic effects. To investigate the synergistic potential of 5-FU-Doxo co-treatment in MDA-MB-231 cells, the cytotoxic and morphological effects of the drug combination were assessed at 72 h following treatment. The MDA-MB-231 cells treated with Doxo and 5-FU demonstrated concentration-dependent growth inhibition, and the combination produced greater anti-proliferative effects than either drug alone (Fig. 2A). A synergistic/near-additive interaction was observed at the effective dose of 50 (ED50; mean CI=0.87±0.11), which transitioned to a mildly antagonistic interaction at ED75 (mean CI=1.21±0.07) and a clearly

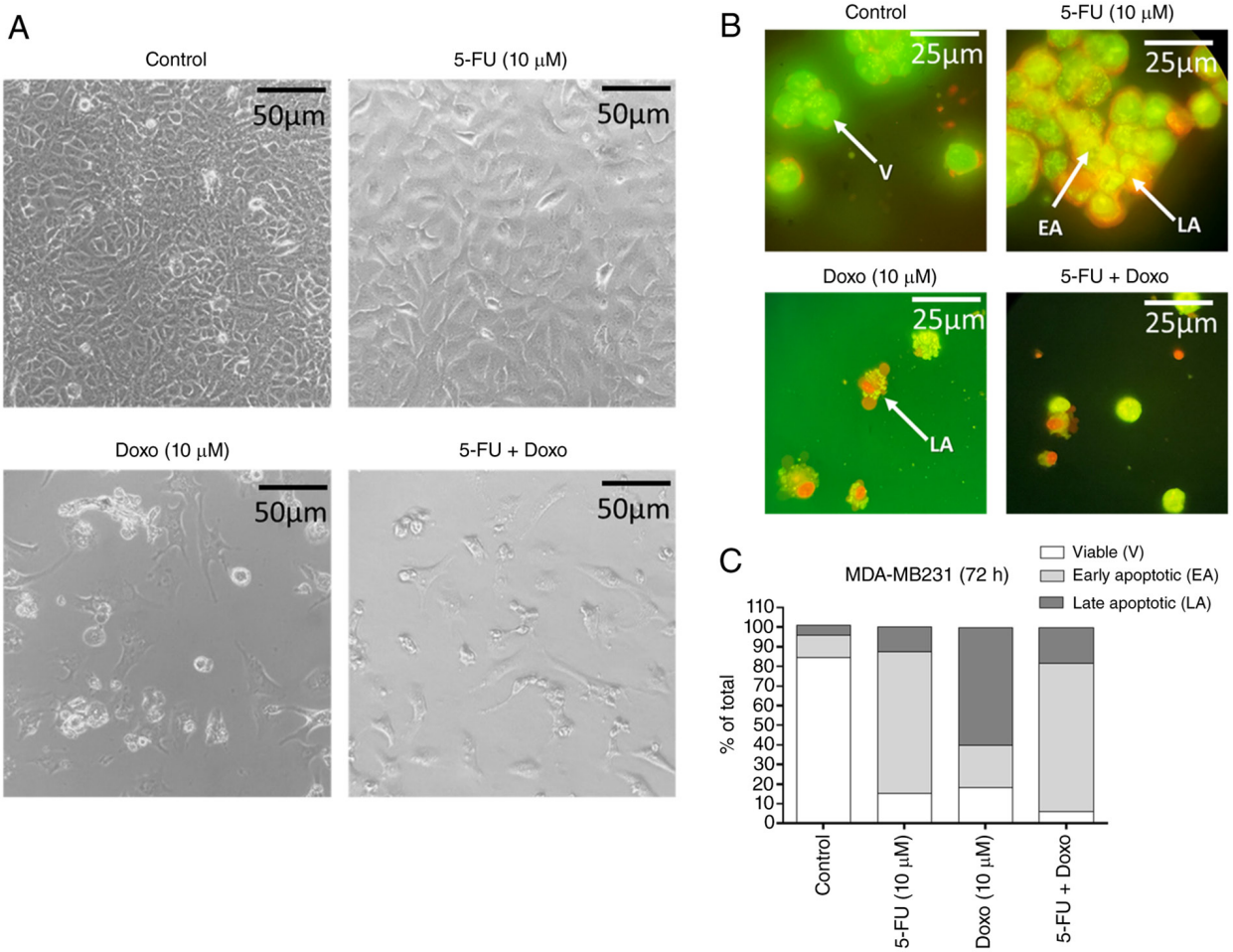

Figure 3. Effects of the combination of 5-FU and Doxo on the morphology and induction of apoptosis in MDA-MB-231 cells. MDA-MB231 cells were treated with control (untreated), 5-FU (10 μM), Doxo (10 μM), or both drugs for 72 h prior to the examination of (A) cell morphology using an inverted microscope at x200 magnification (scale bar, 50 μm), and (B and C) apoptosis using acridine orange/ethidium bromide staining and fluorescence microscopy. (B) Representative images of MDA-MB-231 cells at x400 magnification obtained via fluorescence microscopy (scale bar, 25 μm). (C) Bar chart indicates the percentage of cells in various apoptotic phases per treatment. V, viable (green color); EA, early apoptotic (yellow color); and LA, late apoptotic (orange/red color). Doxo, doxorubicin; 5-FU, 5-fluorouracil.

antagonistic interaction at ED90 (mean CI=2.10±0.33) (Fig. 2B). One-way ANOVA revealed a significant overall difference among the three effect levels (P=0.0020). Tukey's post-hoc test confirmed that the CI value at ED90 was significantly greater than at both ED50 (P=0.0017) and ED75 (P=0.0147), whereas no significant differences were observed between the ED50 and ED75 (P=0.385). The isobologram likewise indicated that the drug combination fell below the line of additivity at ED50, consistent with a synergistic interaction (Fig. 2C). Notably, compared with each agent alone, combination therapy significantly reduced the concentration of Doxo required to achieve the GI_{50} from 8.57 μM to 6.18 μM (a mean reduction of 27.9±19.6%; one-sample t-test vs. a theoretical value of 1, P=0.033) and the concentration of 5-FU from 34.38 μM to 5.00 μM (a mean reduction of 85.5 ± 5.2%; P<0.0001) (Fig. 2D).

Additionally, images captured using an inverted microscope revealed distinct changes in the morphology of the MDA-MB-231 cells at 72 h following co-culture with 5-FU, Doxo, or their combination compared with the untreated cells (Fig. 3A). Notably, treatment with 10 μM 5-FU produced only minor changes in the morphology and number of MDA-MB-231 cells. By contrast, treatment with 10 μM Doxo resulted in a considerable reduction in cell number, accompanied by marked morphological changes, including cell rounding and elongation. Furthermore, treatment with the combination of both drugs led to a further decrease in the number of adherent cells, while increased elongation was observed in the remaining cells (Fig. 3A).

Effects of Doxo plus 5-FU on the induction of apoptosis. AO/EB dual staining and fluorescence microscopy were employed to assess the ratio of apoptosis in MDA-MB-231 cells treated with either the individual drugs or their combination. The cells were cultured with Doxo (10 μM), 5-FU (10 μM) or their combination for 72 h. Representative images of 100 cells, categorized into various stages of apoptosis according to their morphology and color, are presented in Fig. 3B. Notably, the exposure of MDA-MB-231 cells to Doxo monotherapy resulted in ~20% of cells in early apoptosis and 60% of cells in late apoptosis (Fig. 3C). Similarly, the overall level of apoptosis in the cells co-incubated with

5-FU was comparable, although the distribution differed, with ~72% of cells in early apoptosis and 13% of cells in late apoptosis. Nevertheless, compared with single-agent treatment, combination treatment slightly increased the overall proportion of apoptotic cells to ~93% (75% early apoptotic and 18% late apoptotic), indicating a modest increase in apoptosis induction (Fig. 3C).

Discussion

TNBC is a highly aggressive subtype of breast cancer that is resistant to the majority of existing therapies; therefore, the repurposing of established chemotherapeutic agents may represent an attractive therapeutic approach (21). Doxo and 5-FU are FDA-approved chemotherapeutics that have been employed as neoadjuvant therapies for breast cancer (e.g., the FAC regimen). Although the Doxo + 5-FU combination has a clinical history in the treatment of breast cancer, *in vitro* data directly quantifying the exact synergistic indices or the dose-sparing ratios for these two drugs in TNBC are limited. The present study aimed to address this gap by demonstrating definitive synergistic interactions (CI <1) and quantifying the dose-sparing ratios of Doxo and 5-FU in MDA-MB-231 breast cancer cells. Repurposing such well-characterized drugs is beneficial because their safety profiles and pharmacological properties are already established, potentially facilitating more rapid clinical translation (21).

In the present study, Doxo and 5-FU exerted synergistic effects on the TNBC cell line, MDA-MB-231, with combination treatment producing a combination index of <1 and reducing the concentration of each drug required to achieve the same anti-proliferative effect (GI_{50}). Notably, the required concentration of Doxo decreased from ~8.57 μM (a single agent) to ~6.18 μM in the presence of 5-FU. Similarly, the 5-FU concentration decreased from 34.38 μM to 5.00 μM in the combined treatment group. These dose reductions enabled both drugs to achieve the same anti-proliferative effect at lower concentrations, although this interaction became antagonistic at higher effect levels (ED90), as discussed above. Likewise, a dose reduction associated with an enhanced effect, reflected by increased apoptosis, was observed in colorectal cancer cells exposed to a combination of 5-FU and Doxo compared with the same cells treated with single drugs (22). Furthermore, a previous study revealed that co-treatment of MCF-7 cells, an ER-positive breast cancer cell line, with a combination of 5-FU and Doxo produced a synergistic effect (CI=0.39) at specific dose ratios (23). Moreover, a recent preclinical study explored the combination of Doxo and 5-FU in TNBC tumor xenografts and reported increased antitumor effects characterized by enhanced metabolic changes; however, it did not determine the combination index or the appropriate effective doses *in vitro* (11). The present study aimed to address this limitation by determining the effective *in vitro* concentrations and CI values of the Doxo + 5-FU combination in a model of a TNBC cell line and may provide improved *in vitro* dose-sparing of the combination recipe that could inform future *in vivo* studies and dosing in future clinical regimens.

In the present study, the increased cytotoxicity of the Doxo + 5-FU combination was supported by AO/EB dual staining and fluorescence microscopy, which revealed a modest increase in the proportion of apoptotic cells. In addition, examination under an inverted microscope revealed typical morphological alterations (e.g., cell shrinkage and cell elongation) that were more pronounced following combination treatment than following treatment with either drug alone. Overall, these findings indicate that, compared with untreated cells, MDA-MB-231 cells exposed to combination therapy exhibited increased apoptosis and more pronounced morphological changes. These observations are consistent with those of a recent polychemotherapeutic study demonstrating that the combination of Doxo and 5-FU suppressed the growth of breast cancer stem cells in both two- and three-dimensional tumoroid models (24).

A plausible mechanistic basis for the observed synergy can be inferred from the established, distinct modes of action of the two agents. Doxo induces DNA damage through topoisomerase II inhibition and intercalation, thereby activating DNA damage response signaling and apoptosis, whereas 5-FU disrupts DNA/RNA synthesis through the inhibition of TS activity and the misincorporation of fluoropyrimidine metabolites (7,8). In MDA-MB-231 cells specifically, 5-FU has been shown to activate caspases and p53, while inhibiting CDK2, directly linking 5-FU exposure to apoptotic signaling in this cell line (25). Moreover, in other breast cancer models, Doxo pre-treatment has been shown to decrease basal TS expression, thereby increasing the sensitivity of cells to subsequent 5-FU-induced cytotoxicity and enhancing the activation of caspase-9- and caspase-8/3-dependent apoptotic pathways (26). Therefore, the synergistic effect observed in the present study may result from the combined effects of Doxo-induced DNA damage signaling and 5-FU-mediated thymidylate stress converging on shared caspase-dependent apoptotic pathways. However, direct evidence of caspase activation, γH2AX/DNA damage markers, or TS expression analysis in MDA-MB231 cells was not obtained and should therefore be investigated in future mechanistic studies.

This reciprocal sensitivity is probably due to differences in the mechanisms of action of the two drugs, which are not necessarily related to cell proliferation. Unlike in several other cell types, the cytotoxicity of Doxo in normal cells is deemed to be largely mediated by reactive oxygen species. This mechanism has previously been shown to underlie the cytotoxic effects of doxorubicin in embryonic fibroblasts (27), a cell type of the same lineage as REFs. By contrast, MDA-MB-231 cells harbor a gain-of-function mutation of the p53 gene that is known to activate the NF-κB pro-survival pathway specifically in response to doxorubicin, thus dampening its anticancer effect (28). The reduced sensitivity of REFs to 5-FU may be explained by an inverse association between 5-FU sensitivity and dihydropyrimidine dehydrogenase expression, although this remains a plausible but untested explanation in the present study (29). Thus, the proposed mechanisms remain speculative in the current system and need to be directly confirmed.

A critical question is whether the *in vitro* effective concentrations determined herein can be achieved in clinical practice. With respect to 5-FU, the effective combination dose (5.00 μM) is well below the reported peak plasma concentration achieved with standard intravenous dosing (Cmax=400 μM) (30), indicating that clinically relevant 5-FU exposure could readily achieve this concentration. For Doxo, however, the effective

combination dose (6.18 μM) modestly exceeds the reported peak plasma concentration (Cmax=5.9 μM) (31), indicating that this concentration may not be readily achievable at peak plasma levels alone under standard dosing. Doxo is known to be extensively distributed in tissues, and the intratumoral concentration can differ from the plasma concentration depending on tumor vascularity and retention, as discussed above; the effective Doxo concentration identified here may therefore depend on cumulative exposure or tissue distribution rather than peak plasma concentration alone. Overall, while the dose-sparing effect of the combination substantially improved the clinical feasibility of 5-FU, the Doxo requirement remained close to, but slightly above, its reported peak plasma concentration, underscoring the importance of the *in vivo* validation discussed above.

In the present study, the shift from a near-additive interaction at ED50 to an antagonistic effect at the ED90 (Fig. 2B) may reflect concentration-dependent changes in the effects of Doxo on the cell cycle in MDA-MB-231 cells. At submaximal concentrations, Doxo increases the proportion of cells in the S phase rather than inducing early G_2M arrest (32), likely enhancing the S-phase-dependent cytotoxicity of 5-FU and thereby explaining the synergy observed at ED50. At near-maximal concentrations, however, the greater DNA damage burden may drive cell death through pathways less dependent on active DNA replication, reducing the incremental contribution of 5-FU and resulting in antagonism at the ED90. This finding is consistent with the findings of previous research demonstrating that the 5-FU/Doxo interaction is highly dose- and ratio-dependent, shifting from synergistic to antagonistic effects outside an optimal range (33). Clinically, this provides support for dosing within the ED50-ED75 range, where synergy and dose-sparing appear to be well maintained.

In addition to plasma pharmacokinetics, the tumor microenvironment presents further challenges that may limit the translation of effective *in vitro* concentrations to *in vivo* efficacy. Several factors have been identified in solid tumors that can impede the diffusion of drugs and hinder the uniform distribution of a chemotherapeutic agent throughout the tumor, including elevated interstitial fluid pressure, a dense extracellular matrix, and heterogeneous vascular perfusion (34,35). Specifically, hypoxia, which is known to occur in poorly vascularized areas of solid tumors, downregulates the expression of topoisomerase II, potentially increasing the resistance of these tissues to topoisomerase II targets, such as Doxo (35). Therefore, achieving plasma concentrations comparable to the effective *in vitro* concentrations identified in the present study does not necessarily ensure equivalent intratumoral drug exposure. The clinical toxicity profiles of Doxo and 5-FU also overlap substantially. Both agents contribute to myelosuppression, which represents a major dose-limiting toxicity both individually and in combination regimens, such as FAC (36,37). Doxo is also associated with a well-established risk of cumulative, dose-dependent cardiotoxicity. As the data of the present study demonstrated that the Doxo + 5-FU combination significantly reduced the dose of each agent required to achieve equivalent cytotoxicity (Fig. 2D), this dose-sparing effect may also reduce the severity of overlapping toxicities, including myelosuppression and cardiotoxicity, in a clinical setting, although this remains to be validated *in vivo*.

Despite its promising *in vitro* evidence of synergy, it should also be noted that the present study suffers from several limitations. First, only a single TNBC cell line (MDA-MB-231) was used in the experiments, and the culture conditions do not reflect the complexity of tumors *in vivo*, where metabolism, drug delivery and the tumor microenvironment all affect treatment outcomes. Furthermore, REFs are rodent, non-breast-derived fibroblasts, and validation in a human breast-derived normal cell line (e.g., MCF-10A) remains a critical direction for confirming clinical translational relevance. Additionally, apoptosis was assessed using AO/EB staining in a single biological replicate. More quantitative approaches (flow cytometry and caspase assays) were not feasible due to current resource and laboratory constraints and therefore remain priorities for future validation. Future studies are required using additional molecularly distinct TNBC cell lines (e.g., MDA-MB-468, BT-549 and HCC1937) to confirm that the observed synergistic and dose-sparing effects can be generalized across heterogeneous TNBC subtypes. The validation of efficacy, dosing scheduling optimization, and toxicity should therefore be established *in vivo* in TNBC models. Particular attention should be given to myelosuppression and cardiotoxicity due to their overlapping toxicity profiles. Nevertheless, the demonstration of synergy at clinically relevant concentrations provides a strong rationale for further translational investigation.

In conclusion, the findings of the present study indicate that Doxo, in combination with 5-FU, exerts a synergistic effect on the cytotoxicity of TNBC cells, reducing the effective doses of both agents to concentrations that are close to, or well below, reported clinical plasma concentration ranges. This dose-sparing effect, together with an increase in apoptosis, highlights the possibility of redesigning and optimizing existing dosing regimens to reduce toxicity without compromising their efficacy. These results provide strong justification for validating this repurposed chemotherapeutic strategy *in vivo* and continuing its development in TNBC.

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Availability of data and materials

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

Authors' contributions

AHA conceived and designed the study, and was also involved in the study methodology, formal analysis, data curation, project administration and study supervision. AHA also contributed to the drafting of the manuscript, and developed subsequent revisions of the manuscript. FML contributed to the study design and formal data analysis, and co-wrote the first draft of the manuscript, which was subsequently reviewed and

edited. SAM provided resources, was involved in conducting the majority of the experiments, contributed to the interpretation of the data, reviewed the manuscript, and assisted with data curation. All authors have read and approved the final manuscript. All authors (AHA, FML and SAM) confirm the authenticity of all the raw data.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Use of artificial intelligence tools

During the preparation of this work, AI tools (Claude, Rubriq) were used to improve the readability and language of the manuscript, and subsequently, the authors revised and edited the content produced by the AI tools as necessary, taking full responsibility for the ultimate content of the present manuscript.

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