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ORIGINAL ARTICLE

2-Methoxyestradiol reverses doxorubicin resistance in human breast tumor xenograft

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Abstract
Purpose 2-Methoxyestradiol (2ME), an endogenous estradiol metabolite, was developed as a novel agent based on its antitumor activity and lack of toxicity. This study was designed to investigate the modulatory effect of 2ME on the antitumor effect of doxorubicin (Dox) in resistant breast tumor xenograft. Resistant MCF-7/Dox cells were implanted subcutaneously in nude mice
Methods Treatment with Dox 5 mg/kg, 2ME 30 mg/kg and their combination continued twice a week for 2 weeks.
Results Following 28 days from starting the treatment with Dox alone, the change in tumor volume from first day of treatment was 455.6 ± 16.2%. Combined Dox and 2ME treatment significantly reduced tumor volume to 20.8 ± 43%. Also, combined therapy resulted in enhanced tumor apoptotic and reduced proliferative activities relative to Dox alone. The apoptotic indices were 0.13 ± 0.03 and 0.75 ± 0.06 in Dox alone and Dox + 2ME groups, respectively. For Dox alone group, expression of the proliferative markers PCNA and Ki_{67} were 0.78 ± 0.06 and 0.63 ± 0.18, respectively. They were significantly reduced to 0.28 ± 0.1 and 0.12 ± 0.1 for their corresponding combined Dox and 2ME group. Interaction analysis clearly indicated that 2ME synergies antitumor, apoptotic and antiproliferative activity of Dox. Examining body weight, hepatic and cardiac histopathology of the different treatment groups revealed no significant signs of toxicity.
Conclusion These findings suggest that 2ME reverses Dox resistance, with benign side effects profile.

Keywords 2-Methoxyestradiol · Doxorubicin · Drug-resistance

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Introduction

The emergence of drug-resistant tumor cells during treatment is one of the major problems in breast cancer chemotherapy. When tumor cells acquire resistance to the anthracyclines, e.g., doxorubicin (Dox), they usually show cross-resistance to other antitumor agents, the so-called multidrug resistance (MDR) phenomenon [11]. In fact, MDR can be considered as one of the major reasons for failure of cancer chemotherapy. A promising approach to circumvent drug resistance is to utilize non-toxic compounds in combination with the known and well-established chemotherapeutic agents. The limited success in treatment with these chemosensitizers such as verapamil may be attributed to unattainable effective plasma concentrations of these agents due to severe adverse reactions. It was, therefore, warranted to search for additional chemosensitizers with a large therapeutic index and true clinical value [24]. The finding that 15–20% of resistant breast cancer patients respond better to second-line therapy with another endocrine agent drew the attention to the

additive role of hormonal therapy [14]. Thus, several studies investigated the use of steroidal hormones for reversal of drug resistance [11, 31].

The estradiol metabolite, 2-methoxyestradiol (2ME), has been identified as a novel antitumor agent combining antiproliferative, antiangiogenic actions on a wide range of tumors and lack of toxicity [22, 25]. The mechanisms responsible for mediating its effects are not fully elucidated; however, different actions such as disruption of microtubule function and altering microtubule stability have been suggested [4]. Despite being a natural derivative of estradiol, 2ME binds poorly to estrogen receptors (ER); therefore, its antiproliferative effects are not mediated by ER [21]. On the other hand, some paradoxical effects of 2ME were reported. 2ME was observed to enhance the tumor growth of ER positive cells in the absence of E2. This mitogenic effect is mainly attributable to 2ME intrinsic estrogenic activity [27]. It was also confirmed that in the presence of E2, 2ME suppressed the E2-induced cell growth and resulted in a decrease in ER α expression level. Accelerated down-regulation of ER α may contribute to growth inhibition in the presence of E2/2ME combinations [30]. A great deal of research efforts has been initiated to explore the role of 2ME in cancer chemotherapy because of these unique biological properties. In one in vivo study, doses up to 75 mg/kg of 2ME p.o. were well tolerated in treated mice [7]. However, other studies reported no antitumor activity for 2ME at comparable dose levels [26, 28].

The combination of antiprolifeative and antiangiogenic agents has the potential for antitumor synergy and reduced likelihood of resistance. Unfortunately, there is scanty of information regarding the effect of the combined administration of 2ME with other compounds currently used for breast cancer treatment. Therefore, the current work was designed to investigate the modulatory effect of 2ME on the antitumor effect of Dox in resistant breast tumor xenograft in nude mice.

Materials and methods

Drugs: cell line and cell culture

Dox as hydrochloride salt and 2ME were purchased from Sigma Aldrich Co. (St. Louis, MO, USA). Human breast cancer Dox resistant MCF-7/Dox cell line was used in this study. It was obtained frozen in liquid nitrogen from the University of Texas M.D. Anderson Cancer Center, Smithville, TX, USA. The tumor cell line was maintained in the University of Texas-Medical Branch, Texas, USA, by serial sub-culturing. The cells were grown as ''monolayer culture'' in RPMI-1640 medium supplemented with 10% heat inactivated fetal bovine serum and 100 units/mL penicillin and 2 mg/mL streptomycin (Sigma Chemical Co.). MCF-7/Dox cells were derived from the drug sensitive MCF-7 cells by stepwise selection with Dox [20]. The resistance phenotype status and mechanisms of the MCF-7/Dox cells were clearly checked and defined in an earlier study of Chen et al. [3]. To maintain the drug resistance phenotype, it was cultured in the presence of 2 μM Dox and passaged for 1 week in a drug-free medium before the experiment. The cells were counted and assessed for viability by trypan blue exclusion and washed twice with PBS before injection.

Animals

Athymic nude mice were used for the MCF-7/Dox xenografts. BALB/c (nu + nu + genotype) mice were obtained from Harlan's Sprague–Dawley (Madison, WI, USA). Animals were maintained in pathogen free environment at the animal care facility, where they were provided with sterilized food and water. They were maintained under a 12 h light-dark cycle. Female mice of 4–6-weeks-old, weighing 20–25 g, were used. All the experiments and procedures used in the animal studies were approved by the Animal Use and Ethics Committee of the UTMB.

Reversal of doxorubicin resistance in the MCF-7/Dox cell xenografts

In our experiment, the model of xenografts of resistant breast cancer MCF-7/Dox cells in nude mice was established following that of Ullmann et al. [29]. In detail, transplantable MCF-7/Dox cells were collected and suspended at a concentration of 10^7/300 μL in serum free RPMI medium. Cell suspension was subcutaneously injected in the mice in their right flanks. When the tumor volume reached 100 mm^3 in size approximately after 1 month following implantation, mice were randomized into four different treatment groups, each of nine mice. These are Group A: control (treated with vehicle only, propylene glycol:tetrahydrofuran (THF), 9:1 v/v), Group B: Dox alone (5 mg/kg), Group C: 2ME (30 mg/kg), and their combination in Group D. The treatment was given intraperitoneally twice a week for 2 weeks. The selected doses and vehicle were based on our pilot study as well as previous reports [8, 10, 31]. Each animal was tagged in the ear and followed individually throughout the experiment. The tumor growth was monitored starting on the first day of treatment and the volume of the xenograft was measured every 4 days. An observer blind to treatment estimated tumor volumes by measuring three-dimensions (*a*, *b* and *c*) and was estimated according to the formula [19]:

$$V = (a \times b \times c) \times \frac{\Pi}{6}.$$

Evaluation of effectiveness

The curve of tumor growth was drawn according to the percentage of change in tumor volume from first day of treatment (day 1) in the different treatment groups.

At day 28, all the animals were sacrificed and tumor tissues from each mouse were excised and stored in 10% buffered formalin for subsequent investigations.

Morphological and histological examination

Tumor tissue samples in the treatment groups were taken for hematoxylin and eosin (H&E) staining and slide preparation. The stained slides were digitally scanned and the images were enhanced using a computer software system for histological examination.

Immunohistochemistry

Immunohistochemical examination of the expression of the proliferation markers: proliferative cell nuclear antigen PCNA and Ki_{67} was used to assess the antiproliferative activity in the different treatment groups. After the mice were euthanized, tumors were removed from each group and fixed in 10% buffered formalin and paraffin processed. The tumor sections were incubated overnight at 4°C with mouse antihuman PCNA (Santa Cruz Co., diluted 1:5,000) and Ki_{67} (Dako Co., UK diluted 1:200) antibodies. Immunoreactions were visualized using a streptavidin–biotin complex method followed by diaminobenzidine reaction. The tumor sections were counterstained with hematoxylin to visualize the nuclei. The immunoreactions were determined by counting the number of positive antigens to total cell number in five high power fields (40×) of each section in a blinded fashion. Three tumors per group were analyzed to take their average.

TUNEL assay of apoptosis

Paraffin sections of tumor xenograft from each treatment group were deparffinized and digested with proteinase K (Dako Co.). Chemicon ApopTag Peroxidase In Situ Apoptosis Detection Kit, (Chemicon Co., MA, USA) was used to detect the DNA breaks in apoptotic nuclei in each tissue section.

Slides were counter-stained with Hematoxylin (Poly Scientific, Bay Shore, NY, USA) before mounting, viewed under an Olympus BX51 microscope and images recorded by a DP70 digital camera (Olympus Optical Co. Ltd; Tokyo, Japan). Apoptosis index was determined by counting the number of apoptotic cells compared to the number of normal viable ones in five high power fields (40×) of each section in a blinded fashion. Three tumors per group were analyzed to take their average.

Calculation of synergistic indices

The method described by Wild et al. [32] was followed to analyze the interaction of Dox and 2ME on tumor volume, cell apoptosis and proliferation. Briefly, the mean value in each treatment group was obtained by dividing the observed value of the treated group (B, C or D) by the corresponding control group (A). The expected value of the combination treatment group was obtained by multiplying the values of Dox (B) treatment group to that of 2ME treatment group (C). For, tumor volume and proliferation analysis, the resultant value of the combination group was decreased, compared to the control group. Thus, the interaction indices were obtained by dividing the expected value by the observed value of the combination group. For apoptosis assessment, the resultant value of the combination group was increased compared to the untreated control group. Thus, the interaction index was obtained by dividing the observed value by the expected value. An index of greater than 1 indicates a synergistic effect; an index of 1 indicates an additive effect, whereas an index of less than 1 indicates an antagonistic effect.

Evaluation of toxicity

The toxicity of the treatment was evaluated in a semi-quantitative manner. Body weights of mice were measured every 4 days for evaluation of the systemic toxicity of the treatments. The change in body weight in day 1 and 28 representing the start and end of the experiment was evaluated and analyzed. Other signs of unwanted toxicity monitored included fur-roughing, shedding, local trauma at the site of injection. Decreases in general animal activity were also examined as general signs of systemic toxicity.

After complete gross necropsy examination, the hearts and livers in all treatment groups were preserved in 10% formalin. Tissue samples from different treatment groups were taken for H&E staining to allow histological evaluation of possible cardiotoxicity or hepatotoxicity of Dox and 2ME combination.

Statistical analysis

Data are presented as mean ± SD. Statistical analysis was carried out using one way analysis of variance (ANOVA) followed by Tukey's test for multiple comparisons. Statistical significance was acceptable to a level of $p < 0.05$. Data analysis was performed using Graphpad Instat software program (version 2).

Results

Evaluation of effectiveness

Tumor growth

The effect of Dox and/or 2ME treatment on in vivo tumor growth is presented in Fig. 1. Data are presented as percentage change of tumor volume from first day of the treatment (Day 1). At the end of the experiment (day 28), Dox treatment did not significantly inhibit the growth of the MCF-7/Dox tumor, indicating that it retained Dox resistance. The change in tumor volume in Dox group reached 455.6 ± 16.2% compared to the control value of 517.3 ± 209%. Mice receiving 2ME demonstrated significant reduction in tumor growth, where the change in tumor volume reached 261.7 ± 248. In addition, when Dox and 2ME were given in combination, their antitumor activities were augmented. The percentage change in tumor volume was reduced significantly to reach 20.8 ± 43.

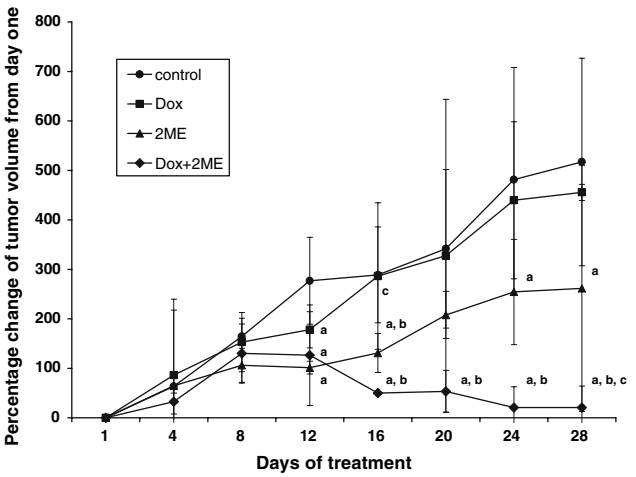

Fig. 1 Antitumor activity of Dox, 2ME and their combination on MCF-7/Dox cells xenograft. When tumors reached 100 mm^3 (designated as day 1), Dox (5 mg/kg), 2ME (30 mg/kg) and their combination were administered i.p. twice a week for 2 weeks. Data represents percentage change in the mean tumor volume from day 1 for each group, $n = 9$. **a** Significantly different from control group at $p < 0.05$. **b** Significantly different from Dox group at $p < 0.05$. **c** Significantly different from 2ME group at $p < 0.05$

Histological examination of tumor tissues

MCF-7/Dox tumor in control group showed a central area of necrosis interspersed with many regions of viable tumor cells near blood vessels and a margin of actively proliferating cells at the tumor periphery (Fig. 2a). Tumor samples treated with Dox showed no observed changes in the pattern of the tumor cells, compared to the control group (Fig. 2b). The tumor cells in the 2ME-treated groups showed vacuolization of their cytoplasm and degeneration of their nuclei. The nuclei shrunk in size and became pyknotic. Some nuclei shifted toward the periphery of the cells (Fig. 2c). Figure 2d shows complete degeneration of the tumor cells in the subcutaneous region with remnant of pyknotic nuclei in the combination group.

Immunohistochemistry of proliferative markers

To determine the antiproliferative effect of 2ME in combination with Dox, immunohistochemical staining with an antibody against PCNA was performed. Most of the cancer cells in the tumor tissue stained positive for PCNA antibodies (Fig. 3a). The proliferative index for Dox group (0.78 ± 0.05) was not statistically significant from that of the control (0.9 ± 0.06) (Fig. 3b). On the other hand, 2ME significantly reduced the expression of PCNA (0.55 ± 0.06) in tumor tissue compared with control group (Fig. 3c). Moreover, the combination of Dox and 2ME significantly reduced the expression of PCNA to reach 0.28 ± 0.1 (Fig. 3d) compared to Dox alone. Figure 3g represents the proliferative indices of the different treatment groups.

Examining the expression of Ki $_{67}$ showed similar results. The proliferative index for the control group was 0.72 ± 0.19 (Fig. 4a). Dox alone did not show any significant decrease in the proliferative index (0.63 ± 0.18) compared to the control (Fig. 4b). However, 2ME significantly reduced the proliferative index to reach 0.34 ± 0.1 (Fig. 4c) compared to the control group. In addition, the combination of Dox and 2ME reduced the expression of Ki $_{67}$ to 0.12 ± 0.1 (Fig. 4d) compared to Dox alone. Figure 4g represents the proliferative indices of the different treatment groups.

TUNEL assay of apoptosis

The number of apoptotic cells in the tumor sections was determined by TUNEL assay and counterstaining with H&E. Dox treatment induced an apoptotic index of 0.13 ± 0.03 (Fig. 5b), which was not significantly different from the control (0.08 ± 0.02; Fig. 5a). 2ME treatment significantly

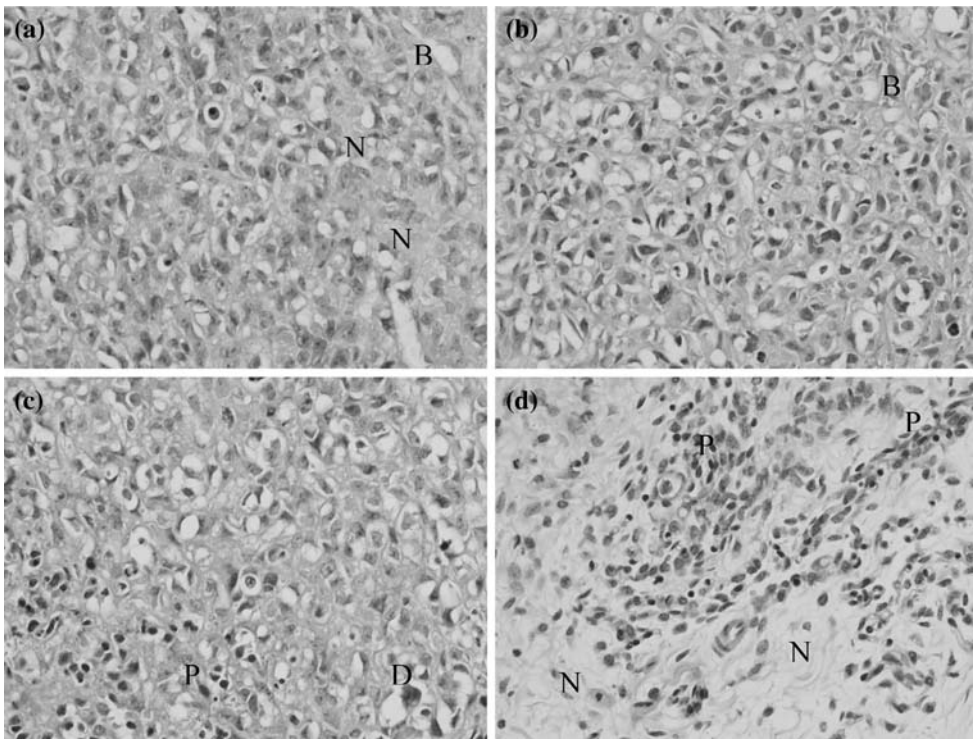

Fig. 2 Histology of representative samples of MCF-7/Dox xenograft in different treatment groups. A sample of tumor in the control group (**a**), Dox group (**b**), 2ME group (**c**) and their combination group (**d**) was excised; H&E stained and photographed (×40). *N* necrotic area, *B* blood vessel, *D* degenerated nuclei, and *P* pyknotic nuclei

increased the apoptotic index to 0.18 ± 0.04 (Fig. 5c), compared to the control group. In the mean while, the combination of Dox and 2ME increased the apoptotic index to 0.75 ± 0.06 (Fig. 5d), which was statistically significant from Dox treatment alone. Figure 5g represents the apoptotic index of the different treatment groups.

Synergistic effects of the combined therapy on tumor volume, apoptosis and cell proliferation

In the current study, the combination treatment resulted in significant synergistic suppression of tumor growth than treatment with single agents individually. Twenty-eight days after starting the treatment, the synergistic indices of tumor volume, cell apoptosis and proliferation by PCNA and Ki_{67} in the combination therapy group were 11, 2.5, 2.46 and 1.7, respectively (Table 1).

Evaluation of toxicity

Body weight

Table 2 presents the body weight of the mice before and after receiving the treatment. The treatment with Dox and/or 2ME resulted in no significant differences in the body weight of the treated animals. The finding indicates a lack of general toxicity caused by the combination.

General signs of toxicity

The regimen of the combination of Dox and 2ME did not cause any deaths in our experimental conditions. None of the tested mice manifested signs of other adverse effects (inactivity, fur-roughing or tissue damage at injection site) as specified in the method section.

Histopathological examination of the heart and liver

No substantial abnormalities were recognized on examining the heart sections of the different treatment groups. Also, no significant differences were detected in the liver sections of the different study groups (data not shown).

Discussion

Development of modulators for drug resistance represents one of the most important strategies in the field of cancer chemotherapy. Unfortunately, the use of drug resistance reversal agents may lead to unacceptable side effects or toxicity [12, 16]. 2ME has several advantages as a drug resistance modulator. It is antiproliferative at doses showing no clinical signs of toxicity [17]. Dox is one of the most valuable chemotherapeutic drugs in breast cancer. Unfortunately, its progress has the price of development of tumor resistance impairing successful therapy. Therefore, the

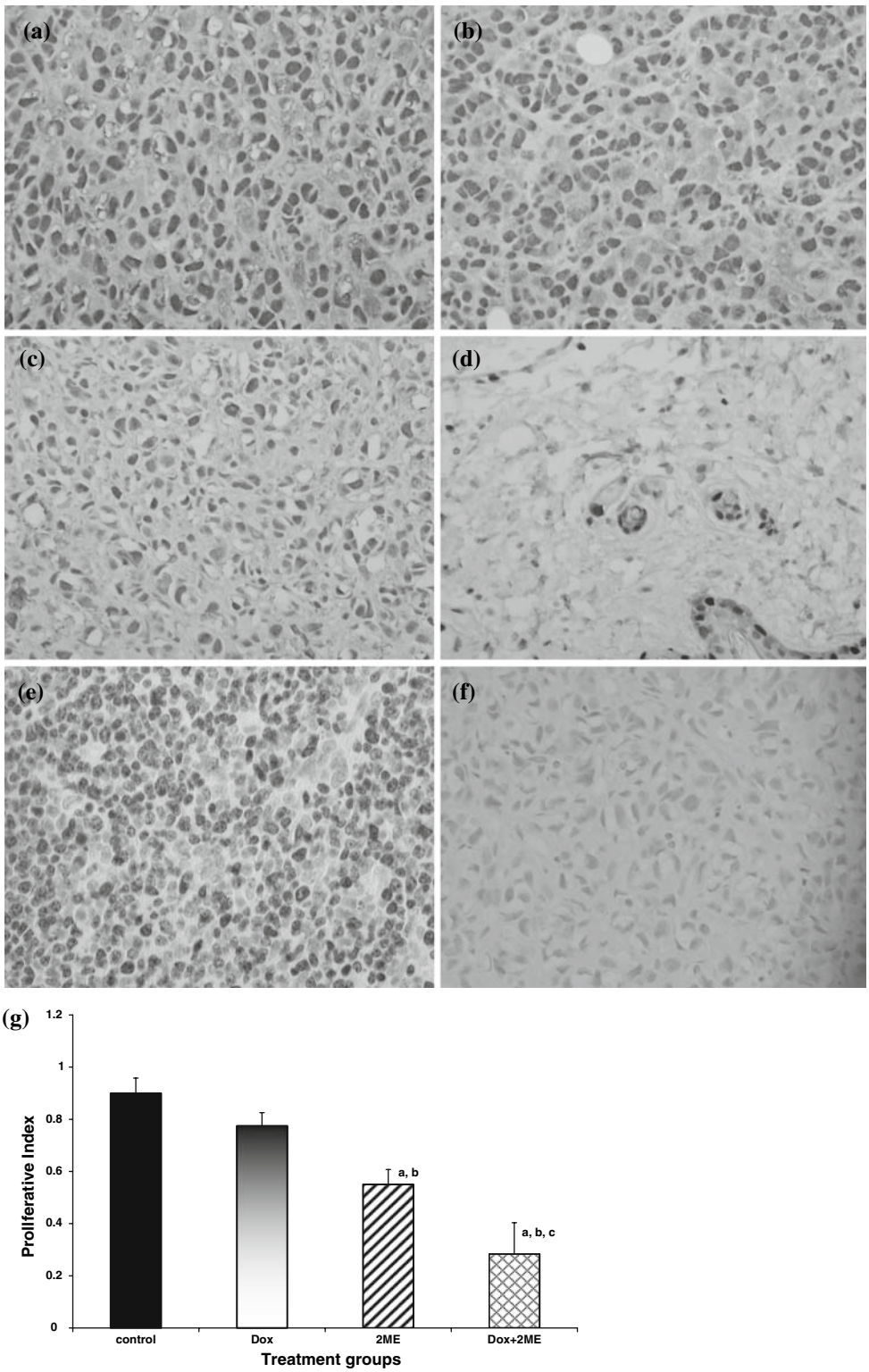

Fig. 3 Representative light micrographs of tumor sections from each treatment group. A sample of tumor in control group **a**, Dox group **b**, 2ME group **c** and their combination group **d** was excised and stained for PCNA antibodies. **e**, **f** Represent positive human tonsil control and negative breast tumor xenograft control, respectively. **g** Represents the proliferative index for each group. *Bars* represent mean ± SD in each treatment group. The mean density for each antigen expression in each treatment group was determined by counting the number of antigen positive reactions in five high power fields (×40) of each section in a blinded fashion. Three tumors per group were analyzed. **a** Significantly different from control group at $p < 0.05$. **b** Significantly different from Dox group at $p < 0.05$. **c** Significantly different from 2ME group at $p < 0.05$

current work was designed to investigate the modulatory effect of 2ME on the antitumor effect of Dox in resistant breast tumor xenograft in nude mice.

Our findings demonstrated that Dox alone did not significantly inhibit the growth of MCF-7/Dox tumor xenograft, as predicted from its resistance phenotype. Our results are in agreement with Mimnaugh et al. [19] who reported that Dox failed to slow the growth of the Dox resistant MCF-7 xenograft. In addition, in the current study 2ME inhibited the growth of the tumor xenograft in treated mice. However, when Dox and 2ME were used in combination, their growth inhibiting activity was significantly

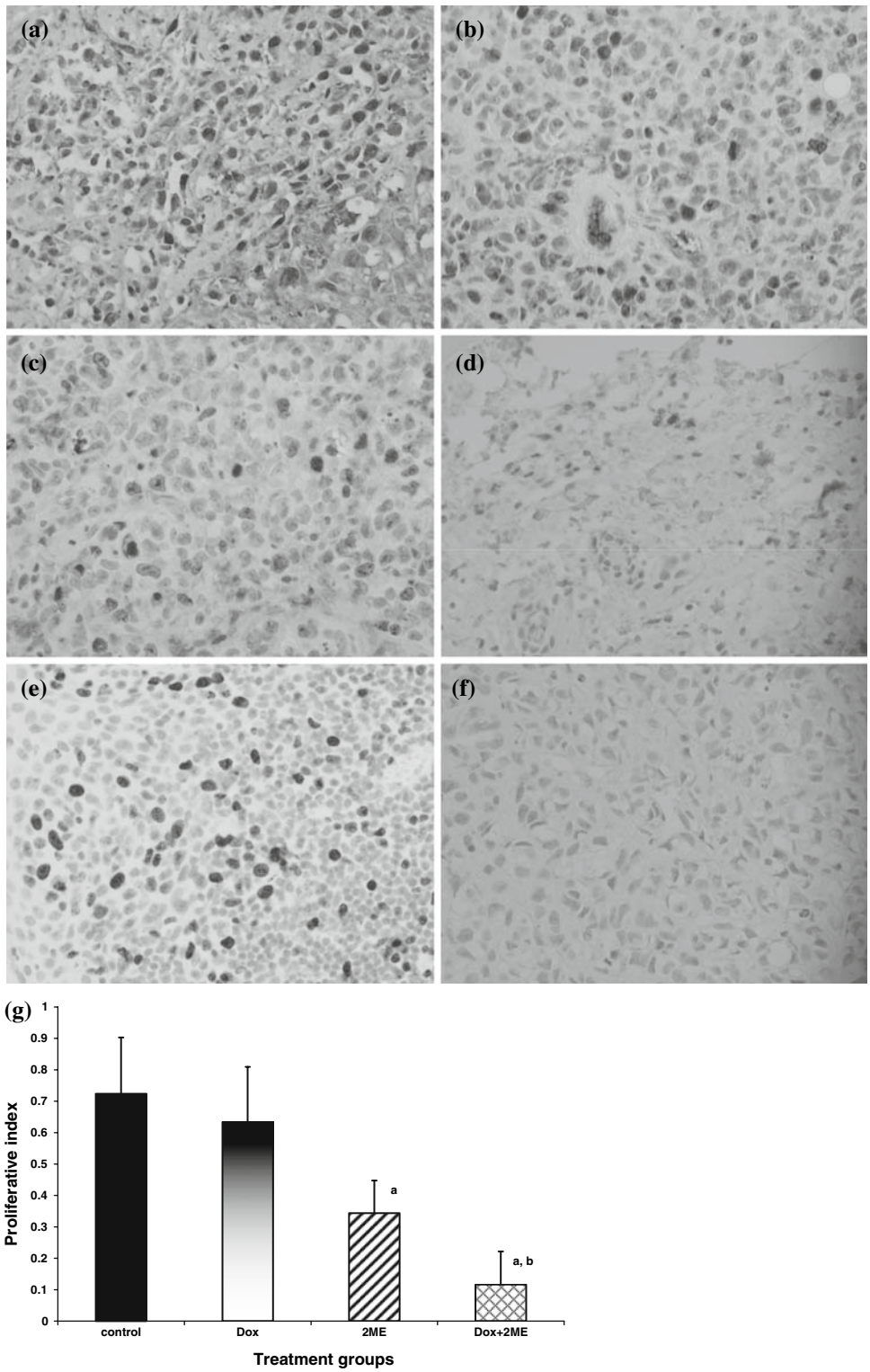

Fig. 4 Representative light micrographs of tumor sections from each treatment group. A sample of tumor in the control group **a**, Dox group **b**, 2ME group **c** and their combination group **d** was excised and stained for Ki_{67} antibodies. **e**, **f** Represent positive human tonsil control and negative breast tumor xenograft control, respectively. **g** Represents the proliferative index for each group. *Bars* represent mean ± SD in each treatment group. The mean density for each antigen expression in each treatment group was determined by counting the number of each antigen positive reactions in five high power fields (×40) of each section in a blinded fashion. Three tumors per group were analyzed. **a** Significantly different from control group at $p < 0.05$. **b** Significantly different from Dox group at $p < 0.05$. **c** Significantly different from 2ME group at $p < 0.05$

augmented. Our work is supported by studies showing that 2ME inhibits in vivo growth of xenografts derived from human breast cancer cells and multiple myeloma cell line [5, 13]. Further, 2ME markedly enhanced the activity of paclitaxel and vinorelbine against the growth of the human breast cancer xenografts [8]. On the other hand, 2ME was found ineffective in inhibiting the xenograft tumor growth derived from melanoma cancer cells [10]. This may be attributed to differences in experimental conditions.

The enhanced antiproliferative effect of the combined treatment was further substantiated by assessing the expression of PCNA and Ki_{67}. Dox treatment alone was not

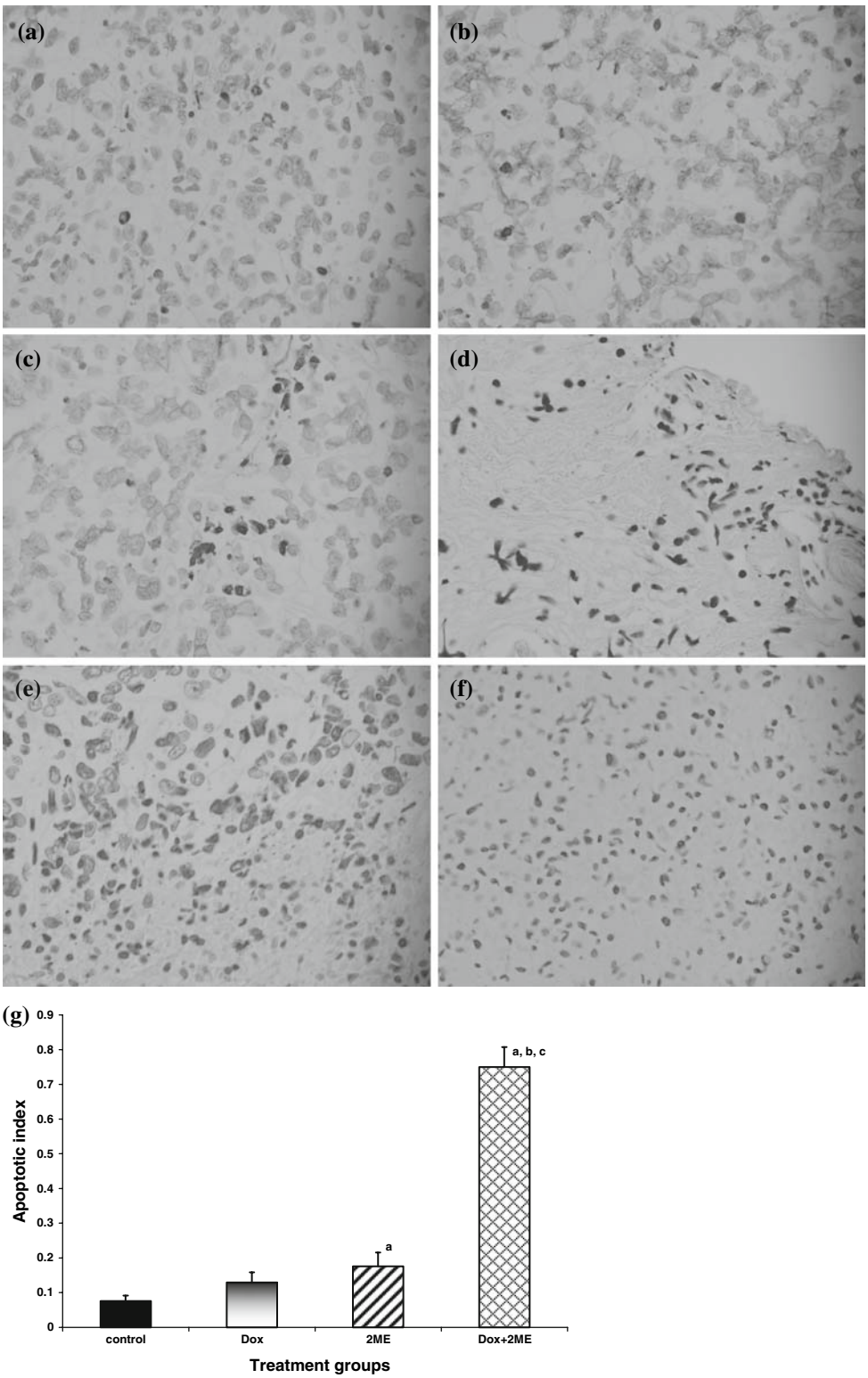

Fig. 5 Representative light micrographs of tumor sections from each treatment group. A sample of tumor in the control group **a**, Dox group **b**, 2ME group **c** and their combination group **d** was analyzed for TUNEL assay of apoptosis. **e**, **f** Represent positive human tonsil control and negative breast tumor xenograft control, respectively. **g** Represents the apoptotic index for each group. *Bars* represent mean ± SD in each treatment group. The mean density for apoptotic index in each treatment group was determined by counting the number of each antigen positive reactions in five high power fields (×40) of each section in a blinded fashion. Three tumors per group were analyzed. **a** Significantly different from control group at $p < 0.05$. **b** Significantly different from Dox group at $p < 0.05$. **c** Significantly different from 2ME group at $p < 0.05$

different from the control group. This confirms the observed ineffectiveness of Dox against the studied tumor model. However, 2ME showed a significant decrease in PCNA and Ki_{67} expression. Combination of Dox and 2ME resulted in augmented reduction in the expression of both markers. Similarly, TUNEL assay of apoptosis indicated that this combination significantly increases the apoptotic index, compared to Dox alone treatment. Interaction analysis revealed that the combination of Dox and 2ME was synergistic in inhibiting tumor growth, inducing

Table 1 Synergistic indices of combination therapy relative fraction

	Dox RF[a]	2ME RF[a]	Combination therapy		Index[c]
			Expected[b]	Observed	
Tumor volume	0.88	0.5	0.44	0.04	11
Apoptosis (TUNEL assay)	1.69	2.3	3.9	9.9	2.5
Proliferation (Ki_{67})	0.88	0.47	0.41	0.167	2.5
Proliferation (PCNA)	0.87	0.61	0.53	0.31	1.7

[a] Relative Fraction (RF) = mean value (tumor volume, apoptosis and proliferation index) experimental/corresponding mean value of untreated control on day 28, respectively

[b] RF of Dox × RF of 2ME treatment

[c] Obtained by dividing the expected RF by the observed RF

Table 2 Body weight of tumor-bearing mice treated with (A) negative control; (B) Dox, (C) 2ME and (D) their combination

Treatment group	Body weight (g)	
	Day 1	Day 28
(A) Control	22.13 ± 0.9	22.07 ± 2.5
(B) Dox	21.05 ± 1.4	20.33 ± 1.6
(C) 2ME	20.96 ± 2.6	21.33 ± 2.7
(D) Dox + 2ME	19.53 ± 3	19.8 ± 0.3

Dox was given at a dose of 5 mg/kg, 2ME was given at a dose of 30 mg/kg using vehicle THF: propylene glycol, 1:9. Nude mice were inoculated with MCF-7/Dox tumor in the right flank and received treatment a month later. Day 1 represents the day of starting treatment and day 28 represents the end of the experiment

apoptosis and reducing proliferation. Our findings are consistent with previous studies reporting the antiproliferative and apoptotic effects of 2ME in multiple meyloma [2], breast cancer cells [23] and melanoma nude mice xenograft [6].

To further characterize the effect of the combination therapy, toxicity profile was examined. No significant differences were found in the body weight of the treated animals in the different groups. Likewise, no general signs of toxicity or deaths were observed in any of the treatment groups. Several concerns were raised regarding the dose limiting cardiotoxicity of Dox [33]. Histopathological examination of heart sections from animals in the different treatment groups showed no observed signs of toxicity. Our findings might appear contradictory to the reported cardiotoxicity of Dox [34]. However, this can be explained on the basis of less cumulative dose and different dosing schedule of Dox used in the current study. In addition, hepatotoxicity of 2ME has been addressed lately [15]. In the present work, examining liver sections of Dox and/or 2ME-treated mice did not indicate any observable histopathological changes. These results gain support by the work of McCormick et al. [18] who reported a non-hepatotoxic profile of 2ME in rats.

In conclusion, 2ME reverses Dox resistance in human breast tumor xenograft in nude mice. This is evidenced by synergizing its antiproliferative and apoptotic effects without significantly enhancing its toxicity. Drug resistance can be acquired after initial exposure to cytotoxic agents [1]. In addition, other studies have found that resistance modulators such as verapamil and cyclosporine A can induce expression of genes mediating drug resistance [9]. It is important to note that the time needed for expression and inhibition of such genes by resistance reversal agents is controversial. So, if such modulation of the resistance phenotype is desired clinically then, it becomes important to design clinical trials with appropriate duration of 2ME treatment.

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