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Combination of 2-methoxyestradiol (2-ME_2) and eugenol for apoptosis induction synergistically in androgen independent prostate cancer cells

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ABSTRACT

Lack of effective treatment options for the management of hormone refractory prostate cancer (PCA) reinforce the great need to develop novel compounds that act singly or in combination. 2-Methoxyestradiol (2-ME_2) is an endogenous estrogenic metabolite that has been reported to work as an antiproliferative agent in various tumor models including prostate. Recently conducted clinical trial in hormone refractory prostate cancer (HRPC) patients concluded that 2-ME_2 was safe and well tolerated. However this study identified bioavailability of 2-ME_2 as a limiting factor. Here we report the ability of a combination of 2-ME_2 and eugenol (4-allyl-2-methoxyphenol) as an approach for enhancing anticancerous activities in prostate cancer cells. Combining 2-ME_2 with eugenol (i) inhibited growth of prostate cancer cells and induced apoptosis at lower concentrations than either single agent alone; (ii) analysis of the data using combination index (CI) showed CI values of 0.4 indicating strong synergistic interaction; (iii) increased population of cells G_2/M phase by 4.5-fold ($p = 0.01$); (iv) significantly reduced expression of antiapoptotic protein Bcl-2 and enhanced expression of proapoptotic protein Bax. Combination induced apoptosis was not affected in PC-3 cells that over-express or lack Bcl-2 but was associated with loss of mitochondrial membrane potential. Since 2-ME_2 was well tolerated in phase II trail in patients with HRPC; and eugenol is consumed by humans in the form of spices, the combination of 2-ME_2 with eugenol may offer a new clinically relevant treatment regimen. Combining these agents may allow ameliorating any adverse effects of either 2-ME_2 or eugenol alone by reducing their individual concentrations should these two agents be developed for human use.

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1. Introduction

Prostate cancer (PCA) is the second leading cause of cancer-related deaths in men and will account for approximately 28,000 deaths every year with a dramatic increase in incidence with age [1]. Given this and the projected aging of the American population, PCA mortality will become a major problem in the future. Most prostate cancer patients are diagnosed with advanced stages of metastatic disease. Early stage prostate tumors require androgens for growth and survival. Hence androgen-deprivation is a standard therapeutic approach. Androgen ablation therapy successfully shrinks primary and metastatic lesions by induction of apoptosis of androgen-responsive prostate cancer cells. Although as many as 80% of patients respond initially to androgen ablation therapy, the duration of this response in most patients is transient (only 12–18 months) because the recurring tumors grow either in the absence of or low concentrations of androgens. Subsequently these tumors become resistant to traditional chemo or radiotherapy treatments leading to uncontrolled tumor growth. No effective systemic therapy currently exists for such tumors [2].

2-Methoxyestradiol (2-ME_2) is an endogenous non-toxic metabolic by-product of estrogens that is present in human urine and blood [3]. 2-ME_2 has been shown to (i) inhibit endothelial cell proliferation implicating its potential role in angiogenesis; (ii) inhibit the growth of different cancer cells including lung, breast, pancreatic, hepatocellular carcinoma, neuroblastoma, medulloblastoma, melanoma and gastric cancer [3–14]. The *in vivo* efficacy of 2-ME_2 has also been tested in several models including breast, pancreas, lung and prostate [3–15]. Further, the efficacy of 2-ME_2 was evaluated in humans by administering it orally to hormone refractory prostate cancer (HRPC) patients who had failed other treatments including hormone therapy. That study concluded that: (i) 2-ME_2 is safe and well tolerated; (ii) PSA levels declined or stabilized in a minority of patients. However, this trial raised significant questions about the bioavailability of the formulation of 2-ME_2 used in these patients [16]. Given that 2-ME_2 targets tumor cells specifically and possesses promising clinical activity albeit with low bioavailability, we investigated whether

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its efficacy could be enhanced by combining it with other non-toxic agents (multicomponent approach). Such approaches have the advantage of (i) reduced toxicity associated problems; and (ii) use of lower concentrations of individual agents [17]. Recently Torrance et al. [18] showed complete inhibition of polyps using a combination of nonsteroidal anti-inflammatory drug Sulindac and irreversible inhibitor of epidermal growth factor receptor kinase, EKI-569 in APC(Min/+) mice. 2-ME_2 has been shown to exhibit additive effect along with tamoxifen and other chemotherapeutic agents in inhibiting the growth of breast cancer cells [19–21]. However, it is not known whether combining 2-ME_2 with other compounds would exhibit better biological activity in prostate cancer cells. In our effort to understand the structure–activity relationships of the 2-ME_2 molecule to increase its efficacy, we identified the requirement of the presence of adjacent hydroxy and methoxy groups on an appropriately substituted aromatic ring for anticancerous activity (data not shown). One such compound is eugenol (4-allyl-2-methoxyphenol) that is found in reasonable quantities in the essential oils of *Syzygium aromaticum* (clove), *Pimenta racemosa* (bay leaves) and *Cinnamomum verum* (cinnamon leaf) that is commercially available. In addition to its use as a flavoring agent in culinary practices all over the world, it has been used as an antiseptic, anti-bacterial and analgesic agent [22–24]. Ghosh et al. [25] have shown that 125 mg/kg body weight of eugenol caused significant delay in tumor development and no signs of metastasis in B16 melanoma xenograft model. However to the best of our knowledge anticancerous activity of eugenol either alone or in combination was not tested for its use in prostate cancer. In this study, we explored the effects of eugenol alone and in combination with 2-ME_2 in androgen responsive LNCaP and androgen-independent PC-3 and DU145 human prostate cancer cell lines. The combined effect of 2-ME_2 and eugenol were analyzed using the multiple drug equations developed by Chou and Talalay [26]. Evidence obtained in this manner indicates that the biological activity of 2-ME_2 can be enhanced significantly when combined with eugenol. These findings identify a novel combination of agents and warrant detailed studies to investigate the precise mechanism of action as well their ability to prevent prostate cancer in pre-clinical models for successful translation for human use.

2. Materials and methods

2.1. Compounds

The structures of 2-ME_2 and eugenol (4-allyl-2-methoxyphenol) illustrated in Fig. 1 were obtained from Sigma–Aldrich (St. Louis, MO).

2.2. Cell lines

Human prostate cancer cell lines, androgen-responsive LNCaP and androgen independent PC-3 and DU 145 were grown in RPMI medium containing 10% FBS and 100 U/ml of penicillin plus 100 μg/ml streptomycin as described earlier [12,13]. These cell lines differ in the status of androgen receptor (AR), p53, PTEN, estrogen receptors (ERα and ERβ).

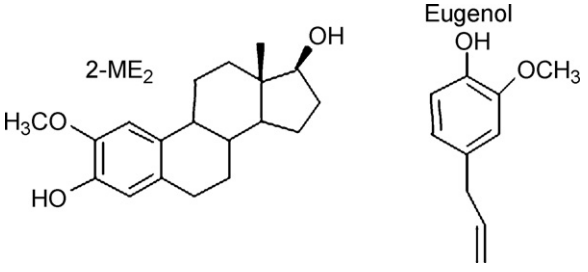

Fig. 1. Structures of 2-ME_2 and eugenol.

2.3. Cell proliferation and cell viability assays

Cancer cells were plated in 96-well plates at a density of 4000 cells per well in triplicate. Following attachment (after 24 h), cells were treated with different concentrations of 2-ME_2 (0.5, 1, 3 and 5 μM) or eugenol (41, 82, 123 and 164 μg/ml) or a combination of both agents. Control cells received only the solvent (DMSO). Cell proliferation was detected after 72 h of incubation using Cell Titer One Aqueous solution assay (Promega Corporation Inc., Madison, WI) as described by the manufacturer [12,13]. Cell viability in the presence and absence of compounds was measured by trypan blue exclusion assay. Briefly following treatment cells were harvested by trypsinization and resuspended in PBS. A small aliquot (10 μl) of cell suspension was added to equal volume of 0.4% trypan blue and viable (unstained cells) cells were counted in a hemacytometer. Cell viability is expressed as the percent viable cells after normalizing to total number of cells in the solvent treated control.

2.4. Treatment of cells

For all biochemical assays including apoptosis, mitochondrial membrane potential, Western blotting and RT-PCR. PC-3 cells were plated at a density of 1×10^5 in 60 mm dishes. At 70–80% confluency, cells were treated with 2-ME_2 alone (0.5 μM), eugenol (41 μg/ml) alone or 2-ME_2 plus eugenol (0.5 μM plus 41 μg/ml) for 24 h unless otherwise mentioned.

2.5. Apoptosis detection

Following treatment both adherent and floating cells were collected by trypsinization for detection of apoptosis using morphological analysis; DAPI (4′-6-diamidino-2-phenylindole) and FITC-Annexin staining as described earlier [27].

2.6. Determination of mitochondrial membrane potential (MMP)

MMP was determined by flow cytometer using The Mitoprobe JC-1 assay kit as per manufacturer's recommendations (Molecular Probes, Inc., Eugene, OR). JC-1, a fluorescent cationic dye 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethyl-benzamidazolo-carbocyanin iodide exhibits potential dependent accumulation in the mitochondria of variety of cells. This is indicated by a fluorescence emission shift from green (~529 nm) to red (~590 nm). Following treatment, cells were trypsinized and 1×10^6 cells were labeled with JC-1 (2 μM) for 30 min in the tissue culture incubator. Following this incubation JC-1 stained cells were pelleted down and washed with PBS and resuspended in 500 μl PBS, and analyzed on a FACScan flow cytometer at the Flow cytometry core facility of University of Texas Health Science Center, San Antonio, TX.

2.7. Preparation of cell extracts and Western blotting

Following treatment cells were lysed in a buffer containing (50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 0.5% NP40, 50 mM NaF, 1 mM Na VO_4, 1 mM phenylmethylsulfonyl fluoride, 25 μg/ml leupeptin, 25 μg/ml aprotinin, 25 μg/ml pepstatin and 1 mM DTT). After passing the lysate through a 25 G needle, cell debris was removed by centrifugation at 12,000 rpm for 30 min. Protein content of the extracts was determined by the method of Bradford as described earlier [11–13]. Equal amounts of extracts were

fractionated on a 10% SDS–polyacrylamide gel. Following electrophoresis, proteins were electrophoretically transferred to a nitrocellulose membrane. The blotted membrane was blocked with 5% non-fat dried milk in Tris-buffered saline containing 0.1% Tween 20 (blocking solution), and incubated with antibodies (Santa Cruz Biotechnology, Santa Cruz, CA and Cell Signaling Technology, Inc. Beverly, MA) followed by incubation with horseradish peroxidase-conjugated anti-rabbit IgG antibody (Sigma) in blocking solution. Bound antibody was detected by enhanced chemiluminescence using Western lightning western chemiluminescence reagent plus (enhanced luminol) following the manufacturer's directions (PerkinElmer Life and Analytical Sciences, Shelton, CT). All the blots were stripped and re-probed with β-actin to normalize protein loading. Each experiment was repeated thrice using different sets of extracts.

2.8. Gene silencing and overexpression assays

PC-3 cells were transiently transfected with Bcl-2 siRNA (100 pmoles) or Bax siRNA (50 pmoles) consisting of a pool of three target-specific 20–25 nucleotide siRNA (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). As a negative control PC-3 cells were transfected with scrambled sequence that does not lead to specific degradation of any known cellular mRNA. Transient transfections were performed as described by us before using lipofectamine [15]. In overexpression studies sub confluent PC-3 cells were transiently transfected with either pCMVBcl-2 or control vector (pcDNA3) using Lipofectamine™ 2000 in triplicate dishes. Knock down of Bcl-2 or Bax or overexpression of Bcl-2 in these cells was confirmed by RT-PCR and protein levels. Following 48 h transfection cells were treated with 2-ME_2, eugenol or combination to determine the effect of Bcl-2 overexpression or silencing on cell viability, apoptosis or mitochondrial membrane potential.

2.9. RNA isolation and RT-PCR

Total cellular RNA was isolated using TRIZOL (Ambion Inc., Austin, TX). Total RNA was reverse transcribed and PCR was performed using One step Access RT-PCR (Promega Corporation, Inc., Madison, WI) as described by us before [25]. Primers for Bcl-2, Bax and GAPDH were obtained from R&D systems Inc., (Minneapolis, MN).

2.10. Colony formation assay

Logarithmically growing PC-3 cells were plated at a density of 13,000 cells/ml in 0.5% agarose plates in triplicate with 2-ME_2 alone, eugenol alone or combination (0.5 μM and 41 μg/ml) as described above for proliferation assays. Plates were prepared fresh by adding 0.5 g agar (FMC 50102) to 100 ml of complete growth media. 0.5 ml of agarose media was evenly layered in 35 mm plates and allowed to solidify for 30 min. 0.5 ml complete media containing 0.5% agar was added to 40,000 cells. After mixing, 0.5 ml media containing cells was poured on top of the 0.5% media in the 35 mm plate. A plate containing no cells was used as a negative control. After 14 days, cells were stained with 0.02% p-iodonitrotetrazolium for 5 h. Colonies that stained dark pink were counted in 10 different fields from each well as described earlier [27].

2.11. Combination index (CI) measurements

Combination index was measured using the method of Chou and Talalay [26], where CI > 1 indicates antagonistic, CI = 1 indicates additive and CI < 1 indicates synergistic interactions.

2.12. Statistical analysis

Data were presented as average ± s.d. and the significance was determined using Student's *t*-test. The differences between the experimental groups was considered to be significant at $p < 0.05$.

3. Results and discussion

3.1. Effect of 2-ME_2, eugenol or combination on cell proliferation

We compared the growth inhibitory activity of 2-ME_2 and eugenol alone and in combination using androgen responsive LNCaP and androgen independent PC-3 human prostate cancer cell lines. The structures of 2-ME_2 and eugenol are shown in Fig. 1. Cells were treated with 2-ME_2 (0.5, 1, 3 and 5 μM) and eugenol (41, 82, 123 and 164 μg/ml) alone and in combination (with escalating concentration of eugenol of 41, 82, 123 and 164 μg/ml while keeping the concentration of 2-ME_2 constant at 0.5 μM) for 72 h using cell proliferation assay. The results are presented as percentage of cell growth following treatment compared to control growth (in the absence of compounds). 50% growth inhibition was observed with 1 μM 2-ME_2 or 82 μg/ml Eugenol in PC-3 cells. Similar level of growth inhibition was achieved using lower concentrations of these agents in combination (0.5 μM 2-ME_2 plus 41 μg/ml eugenol; Table 1). The statistical significance of these observations was determined by ANOVA. As shown in Tables 1 and 2 when combined with eugenol, 2-ME_2 showed a highly significant inhibition of LNCaP cell proliferation at all doses tested; however PC-3 cells showed highly significant inhibition at lower doses used. Under similar experimental conditions, isoeugenol (an isomer of eugenol) had no affect on the proliferation of these cells (data not shown).

3.2. Synergistic effects of 2-ME_2 and eugenol

Graphical representation of the data shown in Fig. 2a indicates that combination of 2-ME_2 with eugenol inhibited the proliferation of LNCaP and PC-3 cells more efficiently than any single agent. These data indicate that 2-ME_2 or eugenol as single agents inhibited the growth of LNCaP or PC-3 cells, however combination of 2-ME_2 with eugenol resulted in a greater growth inhibition at lower concentrations of each compound. The antiproliferative

Table 1
Effect of drugs on LNCaP cell proliferation.

Compound	Dose	*N*	Mean	S.d.	*p* Values
2-ME (μM)	0	13	100	0	
	0.5	13	75.66	8.05	
	1	13	55.53	16.38	
	3	13	53.27	13.56	
	5	3	52.30	16.62	
Eugenol (μg/ml)	0	13	0		
	41	13	78.28	9.21	
	82	13	60.70	21.54	
	123	13	38.96	18.17	
	164	13	21.96	12.46	
2-ME + eugenol					
	0.5 + 41	7	51.20	7.28	0.003
	0.5 + 82	7	22.98	4.37	0.0002
	0.5 + 123	7	22.31	4.17	0.0003
	0.5 + 123	7	38.10	8.70	0.09

Effect of 2-ME alone (0.5–5 μM); eugenol alone (41–164 μg/m) and 2-ME (0.5 μM) with escalating doses of eugenol (41–164 μg/ml) on cell proliferation of androgen responsive LNCaP cells. % Cell proliferation was calculated by measuring normalizing cell proliferation in the presence of treatment with the untreated control. ANOVA model of dose by compound was used to get *p*-values on combination vs single agent by dose.

Table 2
Effect of drugs on PC-3 cell proliferation.

Compound	Dose	*N*	Mean	S.d.	*p* Values
2-ME (μM)	0	4	100	0	
	0.5	4	97.49	3.05	
	1	4	86.73	8.78	
	3	4	64.63	7.95	
	5	3	44.96	7.94	
Eugenol (μg/ml)	0	4	0		
	41	4	73.92	26.66	
	82	4	28.71	25.46	
	123	4	12.32	0.09	
	164	4	12.57	1.35	
2-ME + eugenol					
	0.5 + 41	4	21.86	4.35	0.0002
	0.5 + 82	4	25.44	8.23	0.01
	0.5 + 123	4	45.29	37.19	0.98
	0.5 + 123	4	34.47	18.63	0.63

Effect of 2-ME alone (0.5–5 μM); eugenol alone (41–164 μg/ml) and 2-ME (0.5 μM) with escalating doses of eugenol (41–164 μg/ml) on cell proliferation of androgen independent PC-3.

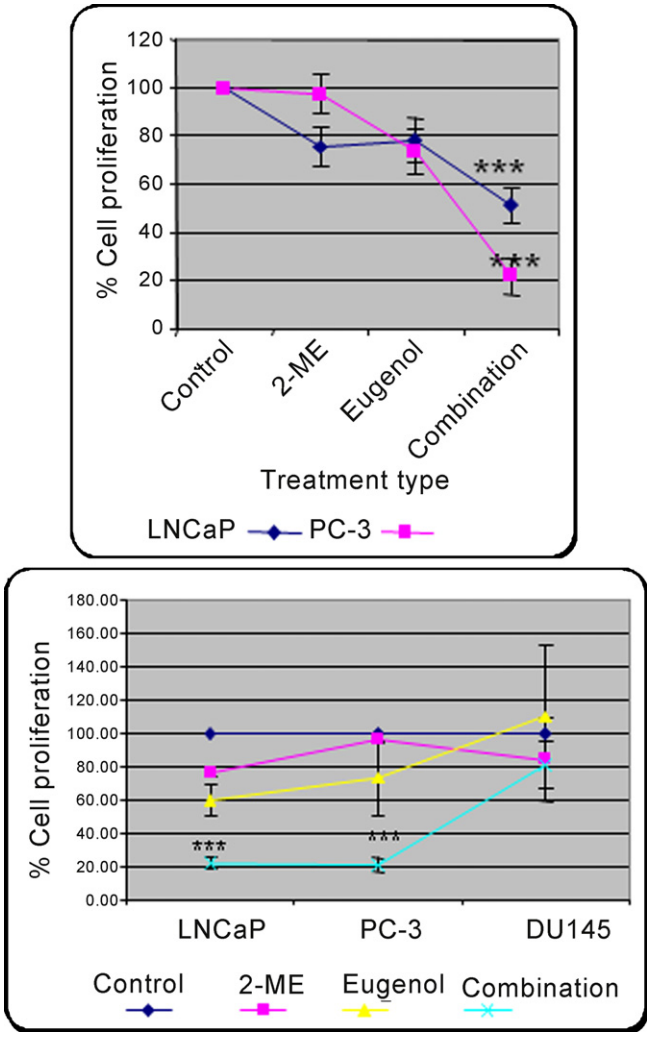

Fig. 2. Effect of 2-ME_2, eugenol or combination on cell proliferation. Androgen responsive (LNCaP) and androgen independent (PC-3 and DU145) cells were plated in 96-well plates as described in Section 2 and treated with 2-ME_2 (1 μM), eugenol (82 μg/ml) or combination of 2-ME_2 + eugenol (0.5 μM and 41 μg/ml). Cell proliferation was measured by Cell Titer96 aqueous one solution assay after 72 h by determining the absorbance at 570 nm using SpectraMaxPlus plate reader (Molecular Devices). Absorbance values obtained with untreated cells was set at 100 and % cell growth was calculated. The data shown here is an average ± s.d. of five replicate wells and is a representative of three independent experiments.

Table 3
Synergistic or additive effect of 2-ME_2 in combination with eugenol as determined by isobologram analysis.

2-ME_2 (μM)	Eugenol (μg/ml)	Avg. CI	S.d.
0.5	41	**0.57**	0.08
0.5	82	**0.70**	0.27
0.5	123	**0.77**	0.07
0.5	164	1.35	0.13
1.0	41	**0.66**	0.17
1.0	82	**0.64**	0.20
1.0	123	**0.81**	0.08
1.0	164	1.41	0.20
3.0	41	**0.86**	0.16
3.0	82	**0.84**	0.21
3.0	123	**0.80**	0.21
3.0	164	1.47	0.09
5.0	41	1.88	1.71
5.0	82	1.03	0.49
5.0	123	0.78	0.13
5.0	164	1.34	0.31

LNCaP cells were treated with indicated concentrations of 2-ME_2 alone or eugenol alone or in combination for 72 h. Cell proliferation was determined as described in Section 2. The obtained data was analyzed using the method of Chou and Talalay to determine the type of interaction. Combination index (CI) of less than one indicates strong synergism; CI of 1 indicates additive effect and CI of more than 1 indicates antagonism. Strong synergism is indicated in bold. The data shown here is an average ± s.d. of three independent experiments that was conducted in quadruplicate wells.

effects of 2-ME_2 and eugenol combination were analyzed using the method of Chou and Talalay to determine the type of interaction (synergistic, additive or antagonistic) of these agents. This method is most readily applied to dose titration data collected for individual agents alone, and the combination of agents added over a broad range of effective doses. It detects combination index (CI) values where CI > 1 indicates antagonism, CI = 1 indicates additive and CI < 1 indicates synergism. Cells were treated with 2-ME_2 (0.5, 1, 3 and 5 μM) and eugenol (41, 82,123 and 164 μg/ml) alone and in combination for 72 h and their effect on cell proliferation was measured using cell proliferation assay. Analysis of this data (Table 3) show that concentrations of 0.5, 1 or 3 μM 2-ME_2 with escalating doses (41–123 μg/ml) of eugenol showed strong synergistic activity (CI of 0.4); and 1 μM 2-ME_2 and 164 μg/ml of eugenol showed synergistic activity with CI values less than 1.0. However higher concentrations of 2-ME_2 (more than 3 μM) with escalating doses of eugenol showed antagonistic activity with CI values more than 1.0. These data suggest that combination of 2-ME_2 and eugenol at appropriate concentrations has the potential for greater antiproliferative activity. These data are consistent with the published results showing interaction between 2-ME_2 and other chemotherapeutic agents [19–21]. In those studies, 2-ME_2 was shown to exert additive effect with 4-hydroxytamoxifen, epirubicine, daunorubicin, paclitaxel and docetaxel in MCF-7 cells. 2-ME_2 has also been shown to enhance the anticancer activity of Paclitaxel or Vinorelbine in human breast cancer cells both *in vitro* and *in vivo* in a synergistic manner [19–21]. However the reasons for the observed antagonistic activity at higher concentrations are not clear at present. In contrast, combination of these agents did not inhibit the proliferation of androgen independent DU145 prostate cancer cells (Fig. 2b). One difference between PC-3 and DU145 cells is that DU145 cells express wild type PTEN making Akt inactive. Although these data indicate that prostate cancer cells with mutated PTEN gene are more sensitive to combination agents than cells with wild type PTEN, the role for differential activation/inactivation of drug metabolizing enzymes cannot be ruled out. More thorough studies including overexpression and knockdown assays with PTEN are necessary to understand the role of PTEN.

3.3. Effect of 2-ME$_2$, eugenol or combination on cell cycle distribution

Previously, we have reported that 2-ME$_2$ treatment arrests cells in G$_2$/M phase in prostate cancer cells [13]. To determine whether the combination of 2-ME$_2$ and eugenol modulates cell cycle profile similarly, we evaluated their effect on cell cycle distribution in PC-3 cells. Representative histograms of cell cycle distribution following exposure to 2-ME$_2$ (0.5 μM), eugenol (41 μg/ml) alone or combination of both agents for 24 h is shown in Fig. 3. Incubation of PC-3 cells with 2-ME$_2$ or eugenol alone showed a modest increase in G$_2$/M and G$_1$-phase with simultaneous decrease in S-phase population of cells. However exposure to both agents together showed significant increase (4.6-fold; $p = 0.01$) in G$_2$/M population that was associated with decrease in G$_1$ population. These data indicate an added advantage for the combination of agents and is consistent with the cell proliferation data.

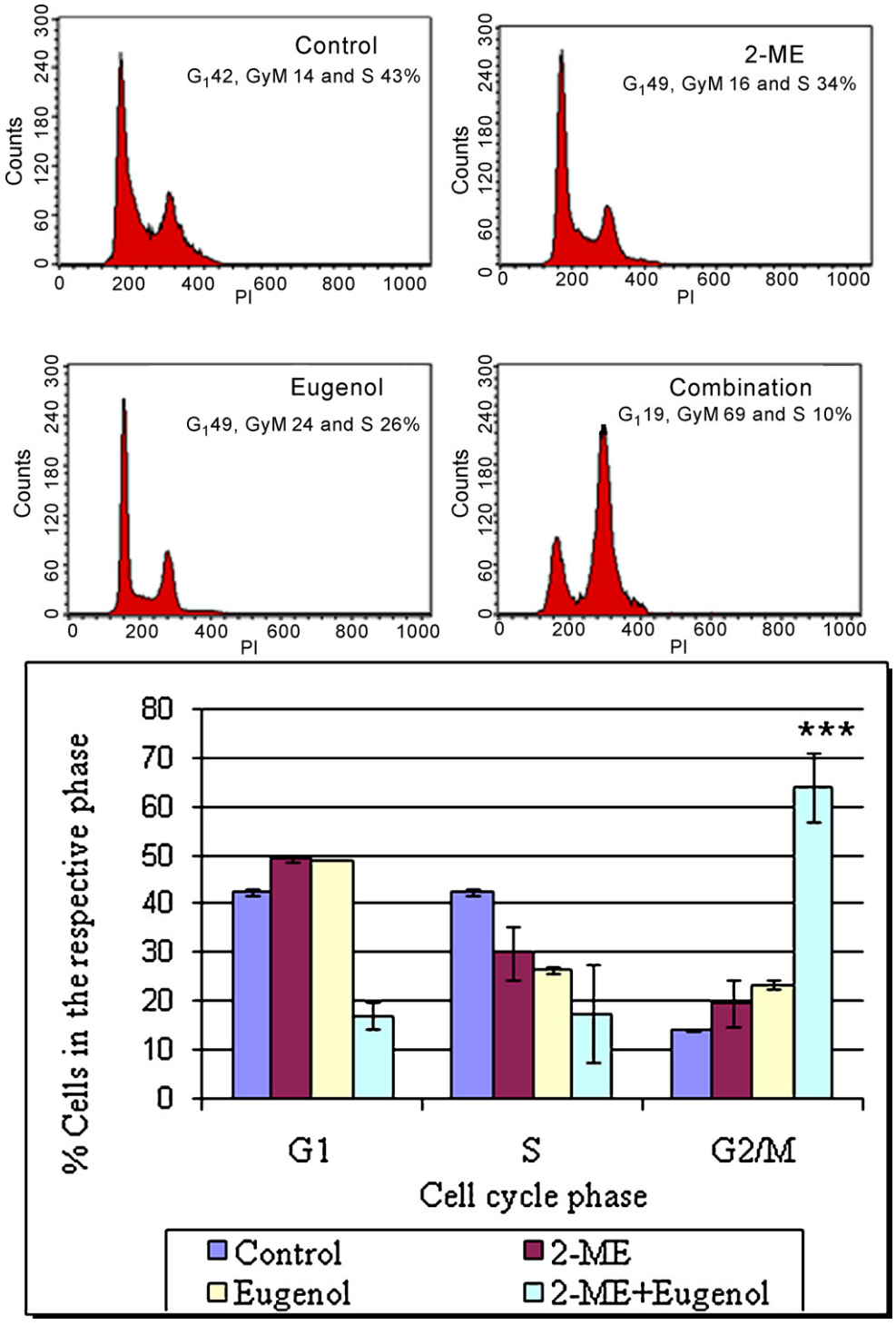

Fig. 3. Effect of 2-ME$_2$, eugenol or combination on cell cycle distribution. PC-3 cells were treated with either solvent alone, 2-ME$_2$ alone, eugenol alone or combination of both agents for 24 h. Distribution of cells in various phases was determined by flow cytometry. Representative histogram is shown in the top panel and graphical representation of the data shown in the bottom panel is an average+ of two independent experiments.

3.4. Effect of 2-ME$_2$, eugenol or combination on morphological alterations and apoptosis in PC-3 cells

Previously, we had shown that 2-ME$_2$ and eugenol induces apoptosis in prostate, neuroectodermal and melanoma cells [11–13]. We investigated the effect of combining 2-ME$_2$ with eugenol on induction of apoptosis in PC-3 cells. Exponentially growing cells at 70–80% confluence were treated with 2-ME$_2$ (0.5 μM) or eugenol (41 μg/ml) alone or in combination for 24 h. Following treatment, significant morphological alterations indicative of apoptosis such as cell rounding and shrinkage, retraction from neighboring cells were observed in the cells treated with 2-ME$_2$, eugenol or a combination of the two compounds (Fig. 4a). Morphological alterations were more evident in the combination group (Fig. 4a). These data prompted us to gather evidence for induction of apoptosis using FITC-Annexin staining. Two-dimensional plots shown in Fig. 4b display green fluorescence (Annexin binding) on the *X*-axis, and orange fluorescence (PI uptake) on the *Y*-axis. Cells in the upper left quadrant with low Annexin and high PI staining represent necrotic cells; cells in the upper right quadrant with high Annexin and high PI staining represent late apoptotic cells; cells in the lower left quadrant with low Annexin and low PI staining represent viable cells and cells in the lower right quadrant with high Annexin and low PI staining represent early apoptotic cells. As shown in Fig. 4b untreated cells show less than 10% spontaneous apoptosis (early and late apoptotic cells); however, incubation with 2-ME$_2$ or eugenol (0.5 μM 2-ME$_2$ or 41 μg/ml of eugenol for 24 h) did not induce apoptosis above background level. In contrast incubation

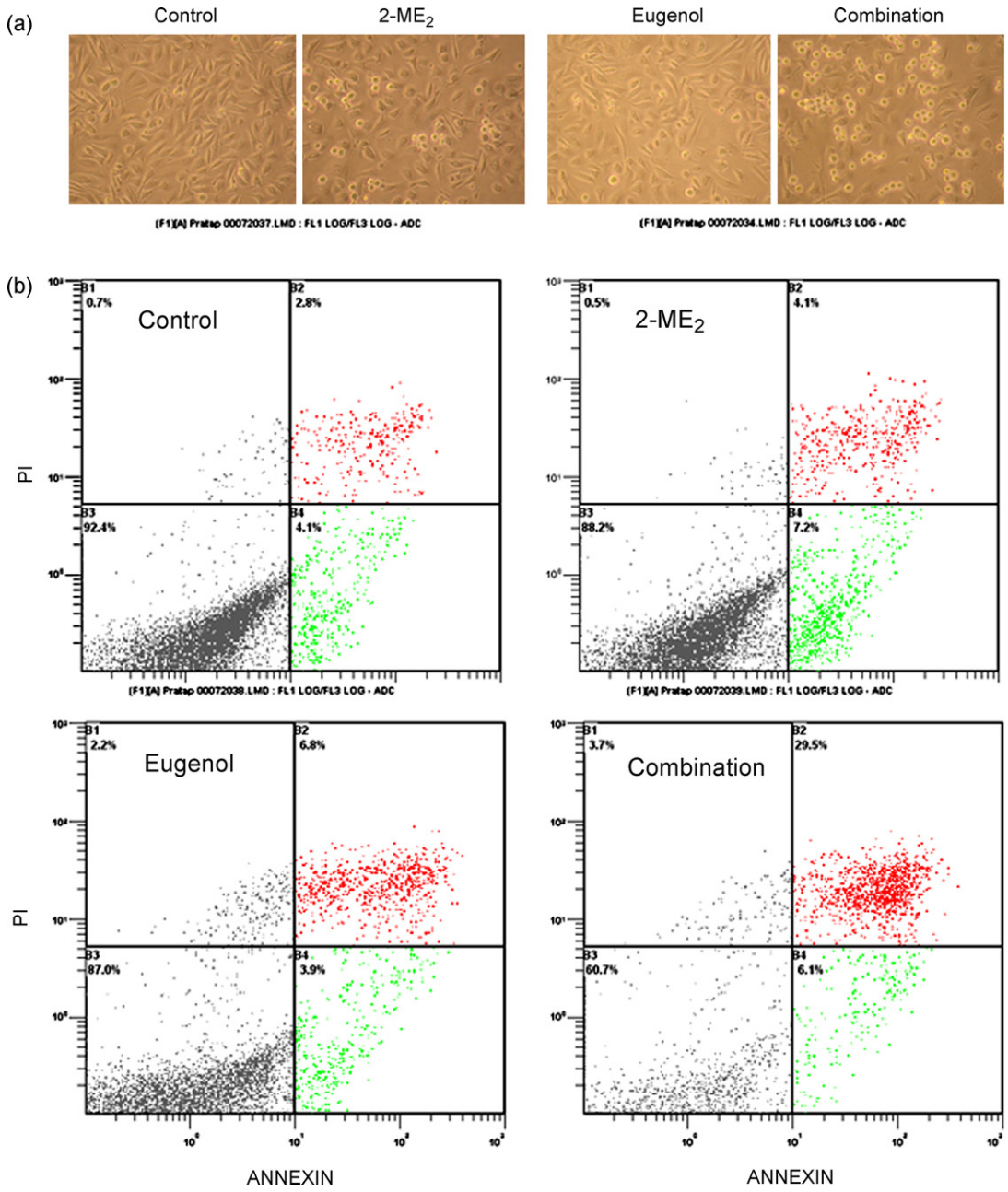

Fig. 4. Effect of 2-ME$_2$, eugenol or combination on morphological alterations and apoptosis in PC-3 cells. PC-3 cells were treated with either DMSO or with 0.5 μM 2-ME$_2$ or eugenol (41 μg/ml) or combination (0.5 μM and 41 μg/ml) for 24 h. Panel A shows photomicrographs of cells by phase-contrast microscopy using a Nikon Microscope with a digital camera system Coolpix 995 at a magnification of 20× (Nikon Corporation, Tokyo, Japan). Panel B shows apoptotic cells as determined by FITC-Annexing staining. A representative histogram is shown.

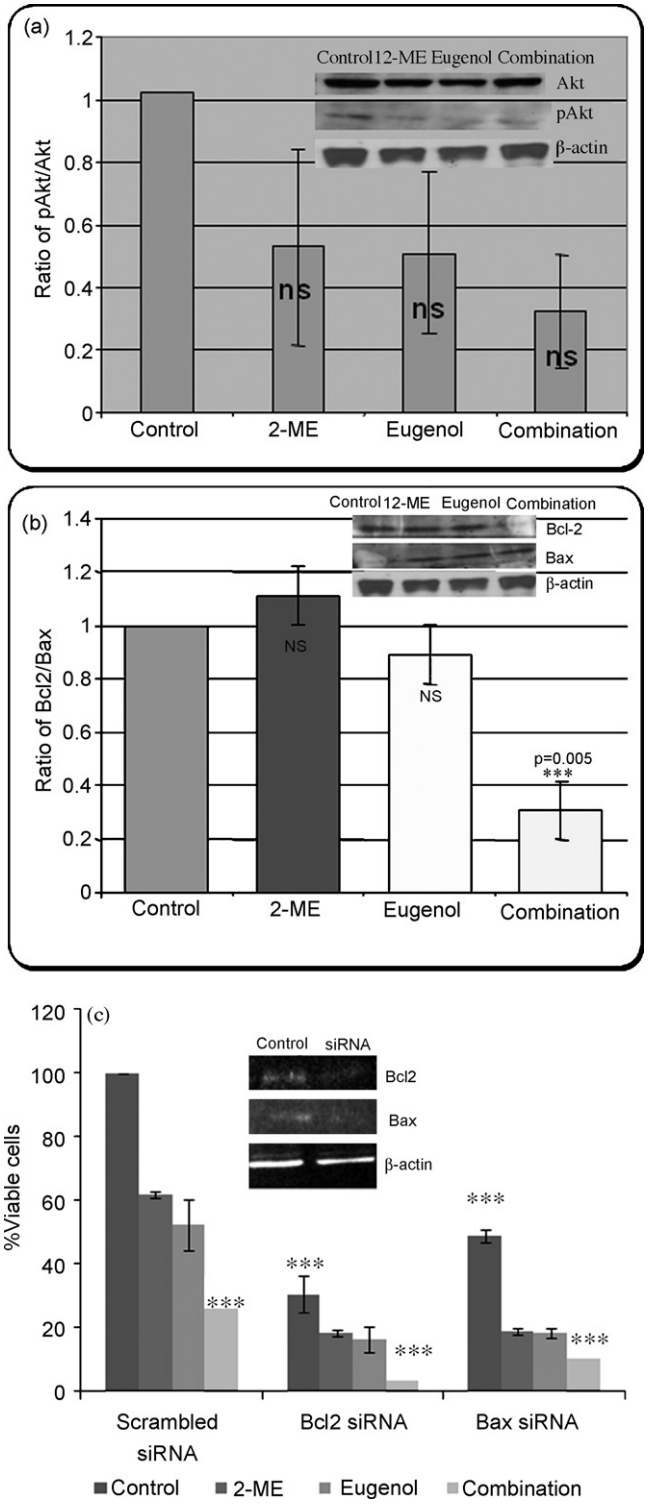

Fig. 5. Effect of 2-ME_2, eugenol or combination on Akt and pAkt (a) Bcl-2 and Bax (b) in PC-3 cells. 25 μg of extract from control or treated cells (as indicated) was fractionated on 10% SDS–PAGE and transferred to a nitrocellulose membrane. After blocking, the membrane was incubated for 2 or 3 h with the indicated antibodies. This was followed by incubation with secondary horseradish peroxidase-conjugated anti-rabbit IgG antibody (Sigma) in blocking solution. Bound antibody was detected by enhanced chemiluminescence using Western lightning western chemiluminescence reagent plus (enhanced luminol) following the manufacturer's directions (PerkinElmer Life and Analytical Sciences, Shelton, CT). All the blots were stripped and reprobed with β-actin to ensure equal loading of protein. Each experiment was repeated thrice using different sets of extracts and a representative blot is shown. Knockdown of Bcl-2 and Bax on the effect of 2-ME_2, eugenol or combination induced inhibition of cell viability in PC-3 cells©. To knockdown Bcl-2 or Bax, PC-3 cells were transfected with Bcl-2 and Bax siRNA or control siRNA as described in Section 2 for 48 h. Following this, cells were treated with solvent control, 0.5 μM 2-ME_2 or Eugenol (41 μg/ml) alone or combination for 24 h. Cell viability was determined by trypan blue exclusion assay. Cell viability is expressed as the % viable cells after normalizing to total number of cells in the solvent treated control. Data from six independent transfections is presented as average ± s.d. Immunoblot analysis of Bcl-2 or Bax in whole cell extracts prepared from transfected cells is shown in the inset. Effect of 2-ME_2, eugenol or combination on apoptosis in PC-3 cells transfected with Bcl-2 (d and e). Sub confluent PC-3 cells were transfected with either pCMVBcl-2 or control vector (pcDNA3) using Lipofectamine™ 2000 (Invitrogen) in triplicate dishes. 48 h following transfection, cells were treated with solvent control, 0.5 μM 2-ME_2 or eugenol (41 μg/ml) alone or combination. Both floating and adherent cells were collected after 24 h of treatment and assessed for induction of apoptosis by FITC-Annexin staining (d). A representative histogram for FITC-Annexin staining is shown in the top panel and graphical representation of the data is shown in the bottom panel (e).

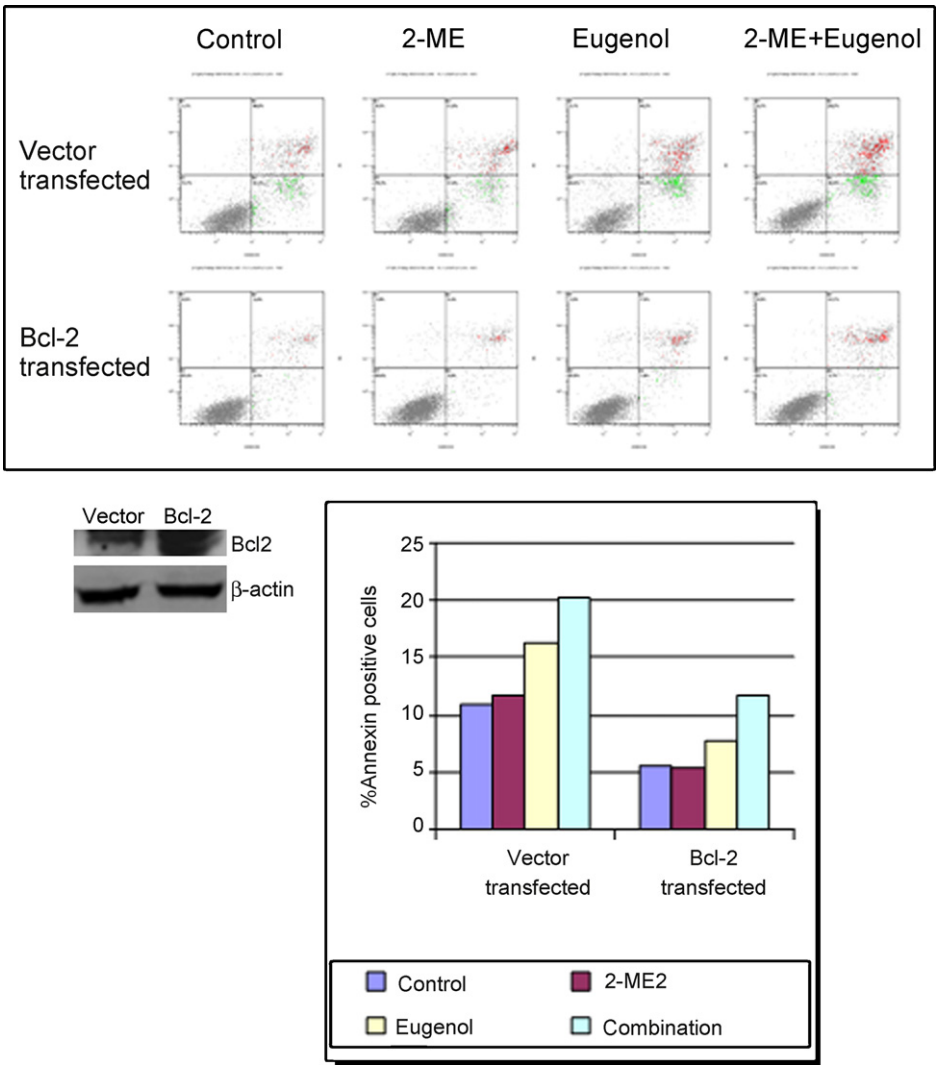

Fig. 5. (*Continued*).

of cells with both 2-ME$_2$ (0.5 μM) and eugenol (41 μg/ml) showed approximately 25% cells undergoing apoptosis. The same concentration of 2-ME$_2$ and eugenol inhibited the growth of both LNCaP and PC-3 cells synergistically. These data collectively demonstrate that this combination of agents inhibits the growth of prostate cancer cells through induction of apoptosis.

3.5. *Effect of 2-ME$_2$, eugenol or combination on Akt and pAkt, Bcl-2 and Bax*

Apoptosis is regulated by interplay between proapoptotic and antiapoptotic proteins of the Bcl-2 family of which Bcl-2 is an antiapoptotic member and is a key regulator of apoptosis [28]. In addition, apoptosis can also be induced by modulating cell survival signaling pathway. Akt, a serine threonine protein kinase represents a key signaling component in the cell survival-signaling pathway. Increased activities of Akt and PI3K (phosphotidylinositol-3-kinase) and mutations in PTEN (a negative regulator of Akt) have been shown to be associated with malignancy and render cells insensitive to apoptosis induction [29]. We investigated whether the combination of 2-ME$_2$ and eugenol induces apoptosis through modulation of antiapoptotic kinase Akt or Bcl-2. As shown in Fig. 5a and b, we did not observe any significant difference in the levels of Bcl-2 or Akt with either 2-ME$_2$ or eugenol as single agents. However combination of both 2-ME$_2$ and eugenol reduced the expression of Bcl-2 significantly. In addition the expression of proapoptotic protein Bax increased in response to the combination agents. Although the protein levels of pAkt and survivin decreased with the combination the observed differences were not significant (data not shown). These data implicate a potential role for Bcl-2/Bax ratio in the combination-induced apoptosis in prostate cancer cells.

3.6. *Involvement of Bcl-2 in 2-ME$_2$ plus eugenol induced inhibition of PC-3 cell viability*

To determine the association of Bcl-2 and Bax with inhibition of PC-3 cell growth and induction of apoptosis, we silenced Bcl-2 and Bax in PC-3 cells. As shown in Fig. 5c, consistent with the above cell proliferation data 2-ME$_2$ plus eugenol inhibited cell viability significantly compared to either compound alone in cells transfected with scrambled siRNA. Consistent with antiapoptotic role for Bcl-2, silencing of Bcl-2 decreased cell viability (compared to untreated scrambled siRNA transfected cells). However 2-ME$_2$ plus eugenol treatment of cells with Bcl-2 or Bax knockdown resulted in further significant reduction of cell viability (compared to 2-ME$_2$ plus eugenol treatment in scrambled siRNA transfected cells). Western blot and RT-PCR analysis was used to determine the knock down of Bcl-2 and Bax (Fig. 5c inset and data not shown). To demonstrate the direct role for Bcl2, PC-3 cells transfected with Bcl-2 were used

in apoptosis assays. As shown in Fig. 5d and e, the combination agents induced apoptosis in these cells over-expressing Bcl-2 as evidenced by FITC-Annexin staining. Similar results were also obtained using Bcl-2 silencing (data not shown). These data show induction of apoptosis by 2-ME_2 plus eugenol in the presence and absence of Bcl-2 suggesting that both Bcl-2 dependent and -independent pathways may be mediating combination induced apoptosis in PC-3 cells.

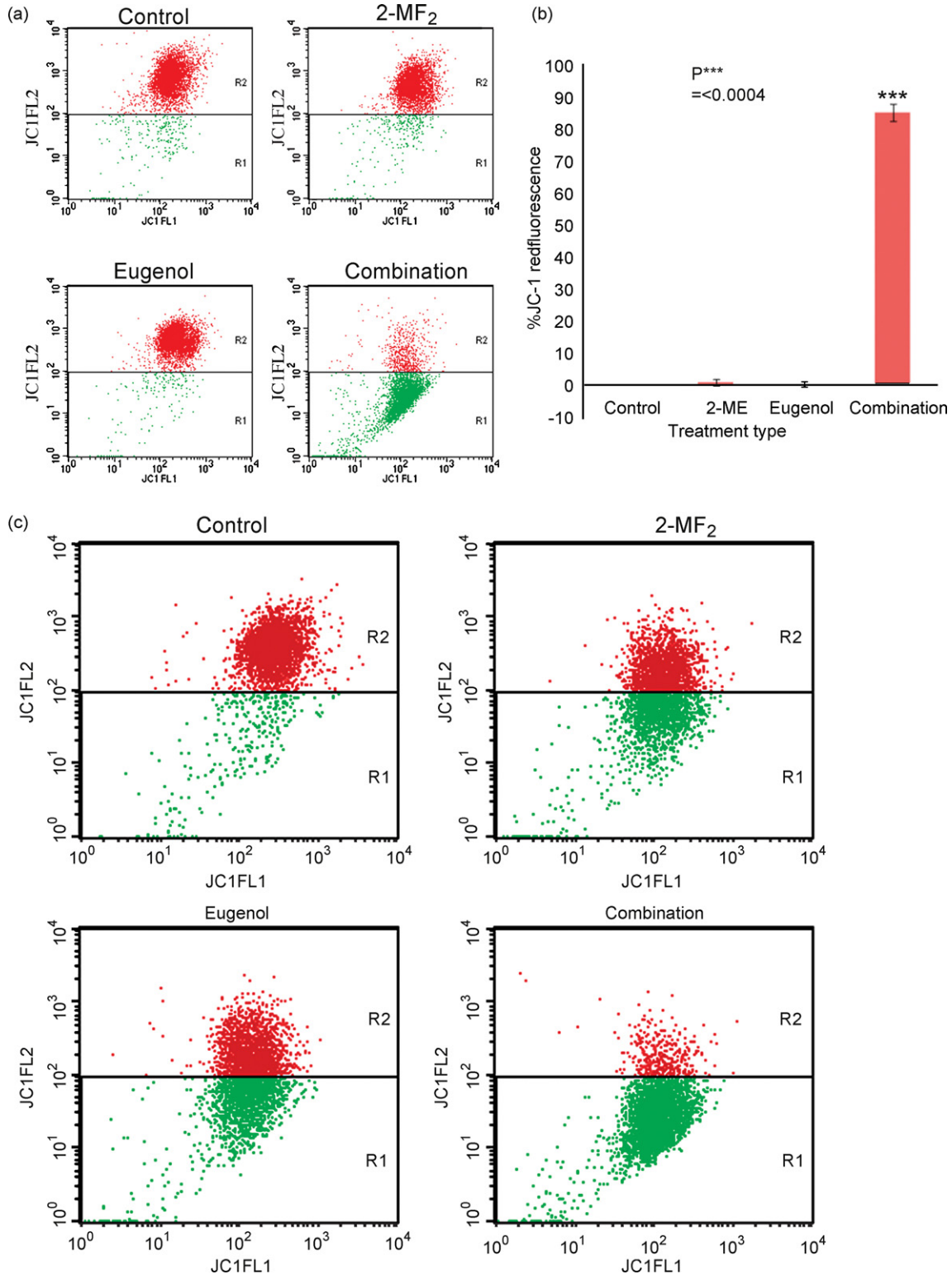

Fig. 6. Flow cytometry analysis of changes of MMP in PC-3 cells treated with 2-ME2, eugenol or combination. Cells treated with the indicated compounds for 24 h were harvested and incubated with JC-1 as described in Section 2. JC-1 stained cells were washed with PBS and analyzed on a flow cytometer using 488 nm excitation with 530/30 and 585/42 nm band pass emission filters. A representative histogram from three independent experiments performed in duplicate is shown in (a). Graphical representation of the data from all experiments is shown in (b). (c) Shows a representative histogram of JC-1 stained PC-3 cells transfected with Bcl-2 siRNA. Scrambled siRNA transfection results were similar to (a).

3.7. Mitochondrial membrane potential in 2-ME$_2$ and eugenol induced apoptosis

Overexpression of Bcl-2 in prostate carcinoma cells is a hallmark of advanced hormone refractory disease [30,31]. Furthermore, in several human tumor cell lines including PCA, Bcl-2 protein expression mediates resistance to the cytotoxic effects of chemotherapeutic agents [30,31]. Such overexpression of Bcl-2 prevents cells from undergoing apoptosis induced by various stimuli through interference with the mitochondrial signaling pathway. Mitochondrial dysfunction has been shown to play an important role in apoptosis through Bcl-2 [32,33]. Such mitochondrial alterations are almost always accompanied by changes in the MMP due to opening of the mitochondrial permeability transition pore. Such changes in MMP can be measured by flow cytometric analysis using JC-1 dye that is specific for mitochondrial potential changes [34]. JC-1 selectively localizes to mitochondria and exists as a monomer at low membrane potential. Under such conditions JC-1 emits at ~529 nm in the green region of the emission spectrum. However under conditions of high membrane potential, JC-1 forms red fluorescent aggregates and emits at ~590 nm. Apoptosis due to changes in MMP can be detected by measuring the ratio of fluorescence intensity (red to green). Healthy cells show a high ratio whereas apoptotic cells show lower ratio [33,34]. We investigated whether alterations of MMP is involved in the combination of 2-ME$_2$ and eugenol induced apoptosis in PC-3 cells. As shown in Fig. 6a, majority of the untreated solvent control cells, 2-ME$_2$ or eugenol alone treated cells showed fluorescence emission at ~590 nm indicating no change in the MMP. In contrast when cells were treated with 2-ME$_2$ plus eugenol, consistently more than 80% of the cells showed fluorescence emission at ~529 nm (fluorescence shift) indicating loss of MMP (Fig. 6a). Similar results were obtained in three different experiments. Quantification of the data (average ± s.d.) is shown in Fig. 6b ($p = 0.004$). These data suggest that a mechanism through which 2-ME$_2$ plus eugenol induces apoptosis in PC-3 cells is through loss of MMP.

3.8. Involvement of Bcl-2 in 2-ME$_2$ plus eugenol induced loss of MMP

We investigated the involvement of Bcl-2 in 2-ME$_2$ plus eugenol induced loss of MMP using Bcl-2 silenced PC-3 cells. We did not observe any significant difference in cells with loss of MMP between scrambled siRNA or Bcl-2 siRNA transfected PC-3 cells following treatment with 2-ME$_2$ plus eugenol (Fig. 6c and data not shown). These data indicate that Bcl-2 may not play a major role in 2-ME$_2$ plus eugenol induced loss of MMP. However about 40% cells showed loss of MMP when treated with 2-ME$_2$ or eugenol alone when Bcl-2 was silenced. These data indicate that Bcl-2 may play a role in 2-ME$_2$ or eugenol induced loss of MMP. However when the two agents are combined, the loss of MMP is increased to 90% indicating that the combination is more effective in disrupting MMP. It has been demonstrated that activation of mitochondria driven pathway is a potential mechanism for induction of apoptosis by several compounds including dichloroacetate, sodium selenite and paclitaxel derivative in prostate cancer cells [35–37]. Our results showing induction of apoptosis through modulation of MMP is consistent with these observations. However the identified combination of non-toxic agents (2-ME$_2$ plus eugenol) is novel.

3.9. Effect of 2-ME$_2$, eugenol or combination on anchorage independent growth of PC-3 cells

Since a characteristic feature of transformed cells is their ability to grow in an anchorage independent fashion, we tested the effect of combination of 2-ME$_2$ and eugenol on growth of colonies in soft agar. As shown in Fig. 7 neither 2-ME$_2$ nor eugenol alone had any significant effect on the number of colonies formed on soft agar. However the number of colonies formed with combination treatment decreased significantly and was consistent with the proliferation inhibition. These data show that the combination inhibits the anchorage dependent and independent growth of prostate cancer cells.

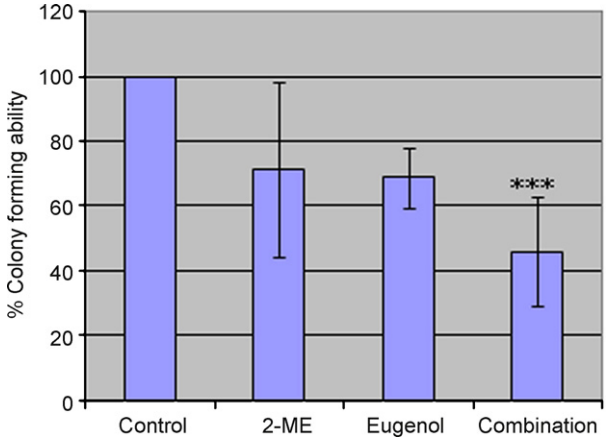

Fig. 7. Effect of 2-ME$_2$, eugenol or combination on anchorage independent growth of PC-3 cells. For anchorage independent growth cells were plated in triplicate in 35 mm dishes on 0.5% agarose containing media as described in Section 2. Following 14-days incubation, cells were stained with 0.5 ml of 0.02% *p*-iodonitrotetrazolium and colonies were counted in 10 different fields from each plate. The results are expressed as mean values + s.d. and is a representative of two independent experiments.

4. Conclusions

Our findings reported here show synergistic inhibition of cell proliferation in highly metastatic androgen independent PC-3 cells that have clinical implications. We also report that the combination with doses lower than the individual agents was effective in inducing apoptosis through Bcl-2 dependent and independent mechanism suggesting the importance of this combination for Bcl-2 resistant prostate tumors. In addition our observations showing lack of effectiveness of the combination in inhibiting proliferation of DU145 cells with wild type PTEN indicate PTEN appears to determine the sensitivity of prostate cancer cells to treatment. These observations are relevant to potential cancer therapies as the PTEN gene is frequently deleted or mutated in prostate cancer. However the role of differential expression and activation/inactivation of drug metabolizing enzymes cannot be ruled out. The mechanism by which 2-ME$_2$ and eugenol synergize to inhibit the proliferation of prostate cancer cells is under further investigation. However because of their significant biological activity in pre-clinical models with no toxic side effects we believe that this combination agent will have a medical as well as an economical impact.

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