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Full Paper

Caspase Mediated Enhanced Apoptotic Action of Cyclophosphamide- and Resveratrol-Treated MCF-7 Cells

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Abstract. Cyclophosphamide (CPA) is a widely used chemotherapeutic drug for neoplasias. It is a DNA and protein alkylating agent having a broad spectrum of activity against a variety of neoplasms including breast cancer. The therapeutic effectiveness of CPA is limited by the high-dose hematopoietic, renal, and cardiac toxicity that accompanies the systemic distribution of liver-derived activated drug metabolites. The present study examines the potential of combining resveratrol (RES) with CPA and aims to increase the understanding of the mechanism of cell killing. Interestingly, we found that RES significantly enhances the caspase-mediated cytotoxic activity of CPA on MCF-7 cells in vitro. RES at 50 μM decreases the IC_{50} value of CPA from 10 to 5 mM. FACS data reveals CPA or RES alone mediated G0/G1 and S phase arrest, while the combination of these drugs released both the arrests and results in an increase in the sub G0/G1 peak. Additional analyses indicated the significant up-regulation ($P = 0.001$) of tumor suppressor p53 and p53-regulated pro-apoptotic Bax and Fas in MCF-7 cells following CPA treatment in combination with RES, which may contribute to the enhancement of the antitumor effect of CPA. Furthermore, downregulation of anti-apoptotic Bcl-2 ($P = 0.001$) was observed in MCF-7 cells treated with CPA with or without RES when compared to untreated MCF-7. These results suggest the possibility of a new combination chemotherapeutic regimen leading to improvements in the treatment of breast cancer.

Keywords: cyclophosphamide, resveratrol, MCF-7, combination chemotherapeutic regimen

Introduction

The concept of combining chemotherapeutic agents to increase cytotoxic efficacy has evolved greatly over the past several years. The underlying rationale is the realization that individual chemotherapeutic agents for the majority of tumors have not increased cure rates in the treatment of cancer (1). However, with the advancements in our understanding of the effects of chemotherapeutic drugs on cancer cells, it is becoming apparent that combination therapy of two or more drugs may have greater beneficial effects on cancer treatment and management.

Cyclophosphamide (CPA) is a cell cycle–dependent DNA and protein alkylating agent that has a broad spectrum of activity against a variety of neoplasms, and it is widely used in the clinical management of human malignancies including breast cancer (2, 3). Furthermore, CPA-mediated p53-dependent apoptosis in MCF-7 breast cancer cells has been reported earlier (4). The beneficial effects of resveratrol (RES) in chemoprevention, cardiovascular disorders, or cancer have been suggested by many studies over the last years to be mediated, at least in part, by its ability to interfere with cell survival programs (5). To this end, RES has been shown to promote apoptosis by blocking expression of anti-apoptotic proteins or by inhibiting signal transduction through the PI3K/AKT, MAPK, or NFκB pathway (5, 6).

We performed the present study in view of the fact that CPA causes auto-induction of CYPs that may enhance the metabolism of RES and hence the combina-

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tion of CPA and RES may have greater beneficial effects on cancer treatment and management. Chang and Wergowske (7) suggested estrogen receptor positive (ER+) status has a beneficial effect in the responsiveness of advanced breast cancer to CPA, methotrexate, and 5-fluorouracil (CMF) chemotherapy and is prognostic of better survival. Therefore, we hypothesize that this combination treatment might enhance the antitumor effect of CPA on ER + MCF-7 breast cancer cells in vitro. We found that CPA with RES significantly enhances the caspase-mediated cytotoxic activity on MCF-7 cells in vitro. Furthermore, normal cell types, that is, non-tumoral cell lines (HEK 293 and MDCK), and other cancerous cell lines (Hep G2 and MDA-MB-231) were also evaluated for cytotoxic effects of drugs. In addition, we explored the molecular basis for the enhanced toxic effect of CPA with or without RES in MCF-7 cells. Our results raise the possibility that this combination of chemotherapeutic regimen may lead to additional improvements in the treatment of breast cancer.

Materials and Methods

Materials

Fetal Calf Serum (FCS) was procured from GIBCO BRL Laboratories (New York, NY, USA); 100-bp DNA ladder from Fermentas Life Sciences (Canada); polyclonal antibody to phosphor-p53 (Ser15), Bcl-2, and Bax and secondary antibodies from Biovision (Mountain View, CA USA). Antibodies against p21Waf1/Cip1, FasL, and sucrose plus protease inhibitor cocktail were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Annexin V-FITC Apoptosis Detection Kit was a kind gift from Dr. Neeraj Sinha. All other chemicals of analytical grade were purchased from Sigma Chemical Co.

Cell culture

MCF-7 and MDA-MB-231(Human Breast Cancer, Epithelial cells: HBCCs), HEK 293 (Human Embryonic Kidney, Epithelial), MDCK (Medin-Darby Bovine Kidney, Epithelial), and Hep G2 (Human Hepatocellular Carcinoma) were procured from the National Center for Cell Sciences (NCCS, Pune, India). These cells are routinely being maintained in our repository and were used in this study (8). Briefly, the cells were cultured in DMEM, pH 7.4 containing penicillin (100 U/ml), streptomycin (100 μg/ml), and gentamicin (60 μg/ml) and supplemented with 10% FCS and 10 mM HEPES in a humidified 5% CO_2 incubator at 37°C. For the experiments, the cells from confluent growth flasks were trypsinized and cultured. Initially for the first 2 days, phenol red-free DMEM containing 10% dextran-coated charcoal stripped FCS (DCC/FCS) was used to deplete the cells of steroids including estrogen present in the serum. DCC/FCS was essentially prepared according to Soto and Sonnenschesin (9). For the subsequent 48 h, the cells were exposed to either the ligands (CPA/RES) or their combination (CPA plus RES). The cell number used and the concentration of ligands exposed to the cells are described for each separate individual experiment. CPA was suspended in PBS and RES was suspended in DMSO.

Cell growth and cytotoxicity assay

3-[4,5-Dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide (MTT) (10) and sulforhodamine-B (SRB) (8) assays were employed to ascertain the cyto-toxic/proliferative effect(s) of ligands in normal cell types (HEK 293 and MDCK) versus Human breast cancer cells (HBCCs) and Hep G2. Briefly, 10^4 viable cells/well were plated in a 96-well plate and exposed to 1 μM to100 mM CPA; 1 μM to 200 μM RES; or various combinations of CPA (1, 5, 10, and 15 mM) with 1, 10, 50, 100, and 200 μM RES. Controls consisted of untreated cells.

Cell-cycle kinetics and apoptosis

To analyze these, 0.2×10^6 cells were plated in a 6-well plate and exposed to 1 – 15 mM CPA (concentrations lower than 1 mM were not cytotoxic), 1 – 200 μM RES, and various combinations of CPA and RES [various concentrations of CPA (1, 5, 10, and 15 mM) of CPA plus 50 and 100 μM RES]. Untreated cells were used as the control. Following trypsinization, the harvested cells were washed with chilled PBS and centrifuged spun at 100 × *g* for 10 min at 4°C. The cells were next permeabilized with 250 μl of chilled 70% ethanol for 30 min at 4°C. Following washing with chilled PBS, the cells were pelleted and resuspended in 500 μl of PBS containing propidium iodide (PI, 40 μg /ml) and RNase (100 μg/ml). Flow Cytometry was performed with a Fluorescence Activated Cell Sorter (FACS) (Beckton, Dickinson and Company, San Jose, CA, USA) employing the Cell Quest software. Cells with DNA content less than that of G0/G1-phase cells were considered to be apoptotic (sub-G0/G1).

Annexin V-FITC and PI staining analysis

For evaluating different stages of apoptosis, 0.4×10^6 cells of each type were plated onto 35-mm culture dishes and treated with 5 and 10 mM of CPA, 50 and 100 μM of RES alone, and 5 mM CPA with 50 μM RES and 10 mM CPA with 100 μM RES in combination. Subsequently cells were washed twice with 1 ml chilled PBS and

stained with Annexin V-FITC and PI according to manufacturer's protocol.

Colorimetric caspase assay

To analyze the role of caspases in CPA/RES- and CPA + RES-induced apoptosis, the MCF-7 cells were cultured and treated in T-75 flasks as described in the previous section. Following scraping, the harvested cells were lysed in lysis buffer (250 mM sucrose, 0.02 M Tris HCl pH 7.4, 1% Triton X-100, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, and 1 mM DTT) at 4°C for 30 min with vortexing. A 200-μg sample of cell lysate protein was mixed in assay buffer (25 mM HEPES pH 7.5, 0.1% CHAPS, 5% sucrose, 5 mM DTT, and 2 mM EDTA) in a final volume of 100 μl, followed by addition of 10 μl of 2 mM of the substrate caspase-8 (Z-IETD-pNA), caspase-9 (Ac-LEHD-pNA), or caspase-3 (Z-DEVD-pNA) for the respective caspase assay. The reaction mixture was incubated at 37°C for 30 min and liberated *p*-nitroaniline (pNA) was measured at 405 nm with a SpectraMAX 190 Microplate Reader (Sunnyvale, CA, USA).

Effects of caspase inhibitor on apoptosis

To determine the inhibition of CPA with and without RES-induced apoptosis of MCF-7 cells, it was estimated in a co-culture with the pan-caspase inhibitor Z-VAD-FMK. MCF-7 cells (0.5×10^6) were preincubated with Z-VAD-FMK (30 μM) for 1 h prior to the addition of 5 mM of CPA with and without 50 μM of RES for 48 h in phenol red–free DMEM (DCC/FCS-treated). The cells were harvested, permeabilized, and stained with PI (40 μg/ml) and analyzed by FACS (Cell Quest Software).

Protein extraction and Western blotting

For western blotting, 4×10^6 MCF-7 cells were cultured in a T-75 flask and treated with 5 mM CPA alone or in combination with 50 μM RES for 48 h. Total cells were harvested washed twice with ice-cold PBS and lysed in ice-cold lysis buffer [0.02 M Tris pH 7.4, 1% Triton X-100, 150 mM NaCl, 1 mM EDTA and EGTA, 1 mM PMSF and DTT, and 0.25 M sucrose plus protease inhibitor cocktail (1 μl/10^6 cells)]. An equal amount of protein samples (50 μg) were resolved on a 10% – 12% SDS-polyacrylamide gel and then electro-transferred onto a nitrocellulose membrane (Amersham, Aylesbury, UK). The membranes were probed with antibodies against Fas, Bcl-2, Bax, phospho-p53 (Ser15), p21WAF/Cip1$^{WAF/Cip1}$, and β-actin. Bound primary antibodies were detected with goat or sheep secondary antibody conjugated to ALP substrate. The immunoblots were detected by using colored substrate (BCIP/NBT).

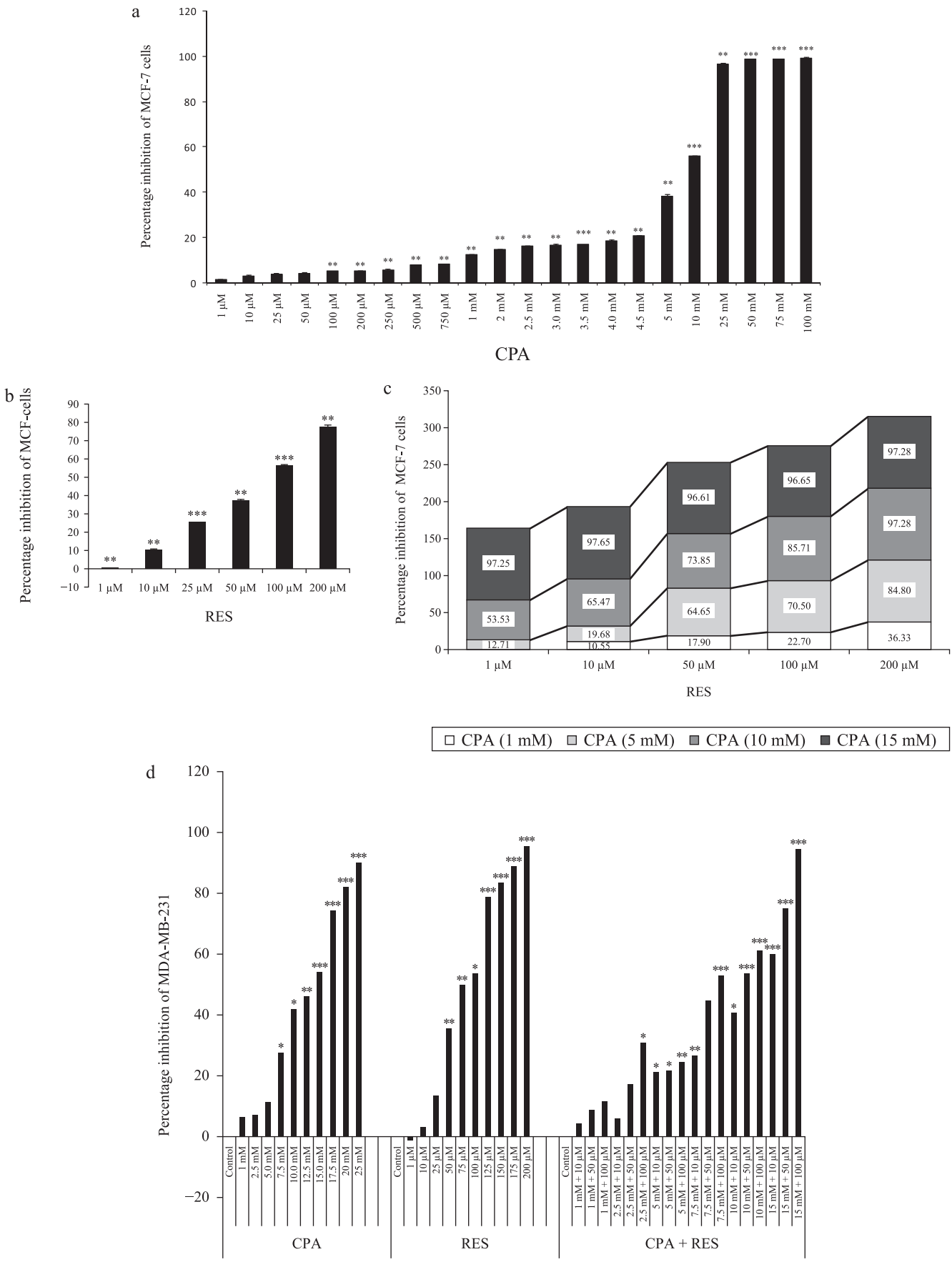
Statistical analyses

All the results are expressed as the mean ± S.E.M. from three similar experiments each performed in triplicate. Student's *t*-test was used to determine the level of significance and a *P*-value <0.05 was regarded as significant.

Results

Regulation of cell growth and induction of cytotoxicity

Normal epithelial (HEK and MDCK) and cancerous (Hep G2) cells were not susceptible to the ligand (CPA/RES/CPA + RES) up to 25 mM CPA, 200 μM RES, and 15 mM + 100 μM CPA + RES (Fig. 1: e – g). In contrast CPA (1 – 25 mM)-treated MCF-7 and MDA-MB-231 exhibited concentration-dependent percent decline in viable cells [through MTT (data not shown) and SRB assays] ranging from 12% – 96% and 6% – 89%, respectively (Fig. 1: a and d). Beyond this range of concentrations (25 – 100 mM), a plateau was reached (Fig. 1: a and d) in HBCCs cells. The IC_{50} of CPA was 10 mM (Fig. 1a) in MCF-7 cells and 12.5 mM in MDA-MB-231 cells (Fig. 1d), which indicates the enhanced susceptibility of MCF-7, unlike MDA-MB-231. RES (1 – 200 μM) caused concentration-dependent percent decline in viable cells ranging from 0 – 77% (Fig. 1b) in MCF-7 cells and 0 – 95% in MDA-MB-231 cells (Fig. 1d). It can be observed that increasing RES concentration resulted in decreased cell viability in MCF-7 and MDA-MB-231. In both cell lines, 100 μM RES was to be the IC_{50} (Fig. 1: b and d). At high concentrations of the molecule (e.g., above 100 μM), cell death was similar in both cell lines (Fig. 1: b and d). Interestingly, at concentrations around 50 μM, MCF-7 appeared to be significantly less sensitive to cell death than MDA-MB-231(Fig. 1: b and d). Taken together, these data suggested that for both cell lines, decreased cell viability and proliferation correlated with cell death at high concentrations of RES. However, at concentrations below 50 μM, diminished cell viability and proliferation was followed by a corresponding increase in cell death in MDA-MB-231 cells (Fig. 1d) but not in MCF-7 cells (Fig. 1b). These differential effects of RES, particularly at low concentrations, could be reflecting a cell-specific alteration of the cell cycle, and eventually, the activation of different mechanisms of cell death. Treatment of MCF-7 with 1 mM CPA together with 1, 10 , 50, 100, and 200 μM RES resulted in modest antiproliferative activity ranging from 0 – 36% (Fig. 1c). CPA the dose of 5 mM together with 1, 10, 50, 100, and 200 μM RES caused a gradual increase in the percentage of non-viable MCF-7 cells (13% – 85%) (Fig. 1c). While at higher concentrations of CPA like 10 and 15 mM with


a
Percentage inhibition of MCF-7 cells
1 µM
10 µM
25 µM
50 µM
100 µM
200 µM
250 µM
500 µM
750 µM
1 mM
2 mM
2.5 mM
3.0 mM
3.5 mM
4.0 mM
4.5 mM
5 mM
10 mM
25 mM
50 mM
75 mM
100 mM
CPA
b
Percentage inhibition of MCF-cells
1 µM
10 µM
25 µM
50 µM
100 µM
200 µM
RES
c
Percentage inhibition of MCF-7 cells
97.25
53.53
12.71
97.65
65.47
19.68
10.55
96.61
73.85
64.65
17.90
96.65
85.71
70.50
22.70
97.28
97.28
84.80
36.33
1 µM
10 µM
50 µM
100 µM
200 µM
RES
CPA (1 mM)
CPA (5 mM)
CPA (10 mM)
CPA (15 mM)
d
Percentage inhibition of MDA-MB-231
Control
1 mM
2.5 mM
5.0 mM
7.5 mM
10.0 mM
12.5 mM
15.0 mM
17.5 mM
20 mM
25 mM
CPA
Control
1 µM
10 µM
25 µM
50 µM
75 µM
100 µM
125 µM
150 µM
175 µM
200 µM
RES
Control
1 mM + 10 µM
1 mM + 50 µM
1 mM + 100 µM
2.5 mM + 10 µM
2.5 mM + 50 µM
2.5 mM + 100 µM
5 mM + 10 µM
5 mM + 50 µM
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CPA + RES

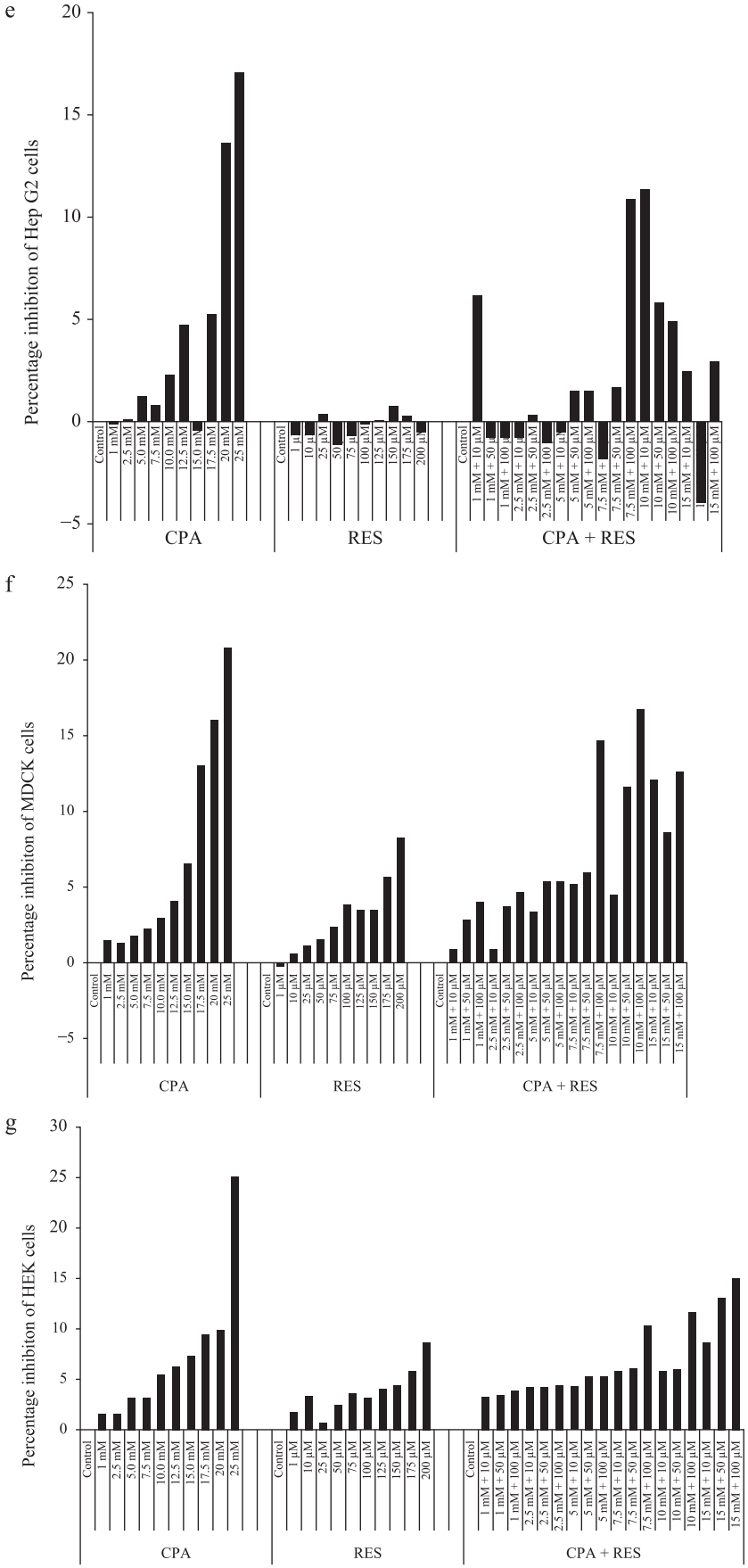


Fig. 1. Cytotoxicity evaluation of cyclophosphamide (CPA), resveratrol (RES), and CPA + RES in normal (HEK and MDCK) versus cancerous cells (MCF-7, MDA-MB-231, and Hep G2). Dose-dependent inhibition of cellular growth of CPA (a)-, RES (b)-, and CPA plus RES (c)-treated MCF-7 cells. Percentage inhibition of cell growth with ligand treatment for MDA-MB-231 (d), Hep G2 (e), MDCK (f), and HEK (g). Cells (0.01×10^6) were pre-cultured for 48 h in phenol red–free DMEM (DCC/FCS-treated), treated with various doses of drugs alone or in combination for 48 h, and IC_{50} values were calculated using SRB Assays. Dose-dependent inhibition was calculated [(Absorbance of control cells) − (Absorbance of treated cells)] / (Absorbance of control cells) × 100. Data shown here are the mean ± S.E.M. of three similar experiments, each performed in triplicate. **P*<0.05, ***P*<0.01, ****P*<0.001 (CPA/RES/CPA plus RES–treated cells as compared to the control).

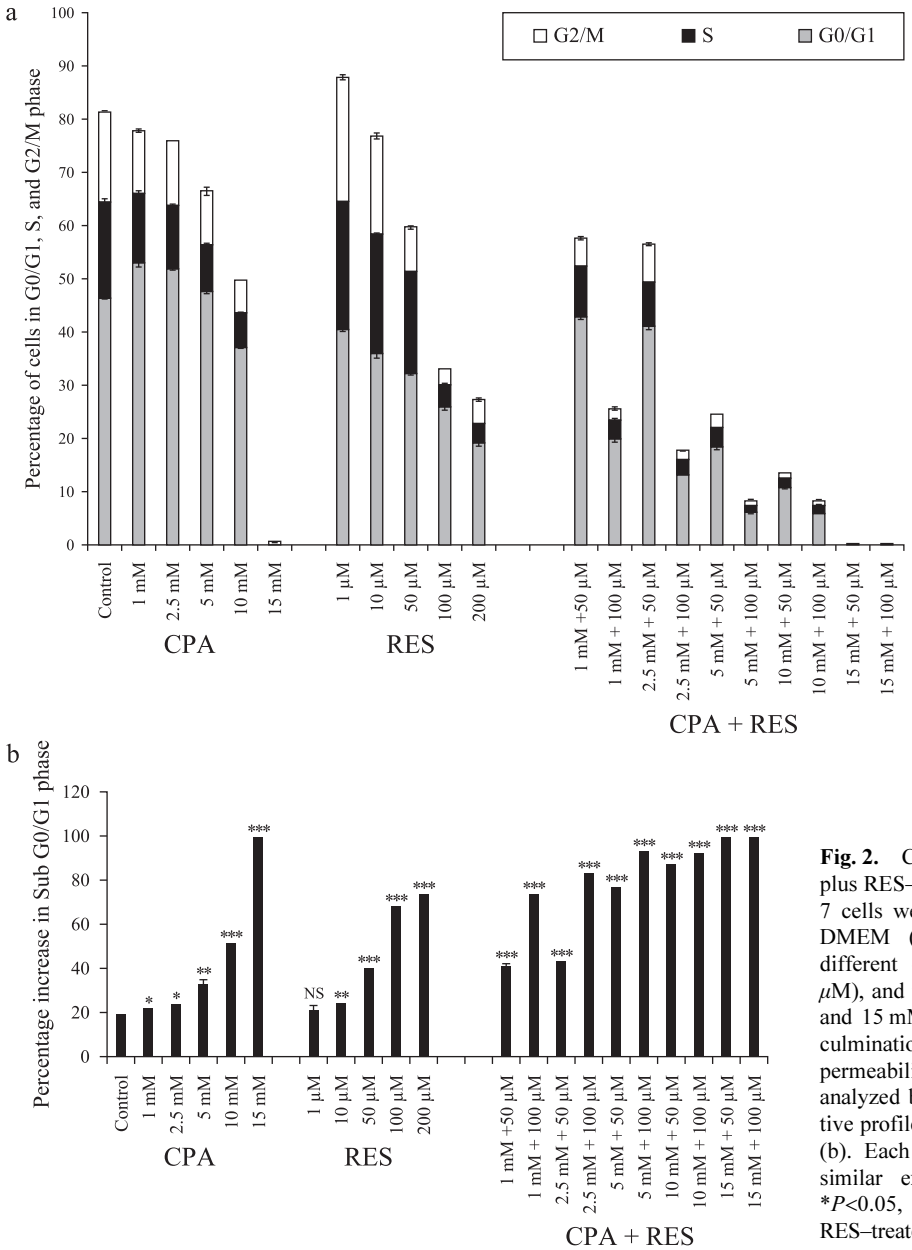


Fig. 2. Cell-cycle analysis of CPA-, RES-, and CPA plus RES–treated MCF-7 cells. Briefly, 0.2×10^6 MCF-7 cells were pre-cultured for 48 h in phenol red–free DMEM (DCC/FCS-treated) and then exposed to different doses of CPA (1 – 15 mM), RES (1 – 200 μM), and various concentrations of CPA (1, 2.5, 5, 10, and 15 mM) plus 50 and 100 μM RES for 48 h. After culmination of incubation, the cells were harvested, permeabilized, and stained with PI (40 μg/ml) and analyzed by FACS (Cell Quest Software). Representative profiles of cell cycle status (a) and subG0/G1 peak (b). Each reported data value is the mean of three similar experiments, each performed in triplicate. *P<0.05, **P<0.01, ***P<0.001 (CPA/RES/CPA plus RES–treated cells as compared to the control).

various combinations of RES (referred above), there was a sharp increase in the percent inhibition of living MCF-7 cells (Fig. 1c). MCF-7 cells treated with CPA at the dose of 10 mM together with RES at all levels showed percentage inhibition of viable MCF-7 cells ranging from 50% – 97%, while at the dose of 15 mM with all the combinations of RES levels, the percentage inhibition of viable cells was 97% (Fig. 1c). The significant observation here is that 5 mM CPA in combination with 50 μM RES is more efficacious in inducing cytotoxicity in MCF-7 cells, so this can be considered as the IC_{50} of the combination. The induction of cytotoxicity in MDA-MB-231 cells by CPA + RES was initiated at 1 mM + 50 μM (Fig. 1d). The highest degree of cytotoxicity (94.09%) was observed at 15 mM + 100 μM CPA + RES (Fig. 1d) and concentrations >7.5 mM + 50 μM CPA + RES were extremely toxic to the MDA-MB-231 cells.

Cell-cycle kinetics and apoptosis

CPA from 1 – 5 mM (52% – 48%) caused accumulation of cells in G0/G1 in comparison to the control (47%), whereas higher concentrations (10 – 15 mM) caused dose-dependent G0/G1, S, and G2/M phase inhibition (Fig. 2a and Table 1). Moreover, in the sub-G0/G1, the apoptotic peak increased significantly at CPA>1 mM (Fig. 2b). CPA induced apoptosis in a concentration dependent manner, reaching values close

Table 1. Cell-cycle analysis of MCF-7 cells following treatment with different doses of CPA (1 – 15 mM), RES (1 – 200 μM), and various concentrations of CPA (1, 2.5, 5, 10, and 15 mM) with 50 or 100 μM of RES for 48 h

Treatment	G0/G1 phase	S.D.	*P* value	S phase	S.D.	*P* value	G2/M	S.D.	*P* value
Control	46.69	0.424264		17.57	0.86267		17.065	0.077782	
CPA 1 mM	52.82	0.494975	0.006131	13.1	0.749533	0.032518	11.915	0.289914	0.017184
CPA 2.5 mM	51.825	0.247487	0.010366	11.79	0.268701	0.048105	12.395	0.007071	0.007019
CPA 5 mM	47.62	0.410122	0.155772	8.61	0.39598	0.018951	10.22	0.735391	0.046026
CPA 10 mM	36.97	0.183848	0.006977	6.57	0.169706	0.02927	5.99	0.014142	0.002298
CPA 15 mM	0.32	0.056569	0.0035	0.055	0.007071	0.022154	0.085	0.007071	0.00189
RES 1 μM	40.51	0.707107	0.016769	23.94	0.084853	0.058584	23.47	0.494975	0.030809
RES 10 μM	35.86	0.848528	0.012412	22.43	0.410122	0.042871	18.58	0.565685	0.158659
RES 50 μM	32	0.282843	0.000703	18.98	0.028284	0.623227	8.32	0.282843	0.010255
RES 100 μM	25.75	0.494975	0.00056	4.32	0.311127	0.015421	2.95	0.070711	3.01E-05
RES 200 μM	19.05	0.707107	0.00155	3.6	0.070711	0.026797	4.685	0.162635	0.001031
CPA + RES (1 mM + 50 μM)	42.99	0.707107	0.03826	9.2	0.141421	0.041257	5.46	0.212132	0.003039
CPA + RES (1 mM + 100 μM)	19.87	0.735391	0.001817	3.56	0.056569	0.027068	2.18	0.212132	0.002219
CPA + RES (2.5 mM + 50 μM)	41.1	0.707107	0.019726	8.2	0.070711	0.040135	7.215	0.304056	0.00902
CPA + RES (2.5 mM + 100 μM)	13.14	0.070711	0.004497	2.895	0.007071	0.026438	1.75	0.141421	0.000382
CPA + RES (5 mM + 50 μM)	18.47	0.636396	0.000869	3.61	0.070711	0.026816	2.475	0.035355	0.000339
CPA + RES (5 mM + 100 μM)	6.155	0.219203	0.000577	1.335	0.035355	0.023682	0.7	0.141421	0.000344
CPA + RES (10 mM + 50 μM)	10.74	0.381838	0.000137	1.845	0.077782	0.023585	1.045	0.06364	2.82E-05
CPA + RES (10 mM + 100 μM)	5.87	0.070711	0.003656	1.45	0.212132	0.017245	1.095	0.120208	0.000145
CPA + RES (15 mM + 50 μM)	0.17	0.014142	0.004064	0.065	0.007071	0.022167	0.045	0.007071	0.001885
CPA + RES (15 mM + 100 μM)	0.135	0.007071	0.004092	0.095	0.007071	0.022205	0.065	0.007071	0.001887

G0/G1, S, G2/M: Percentage of cells in various phases of the cell-cycle. Sub-G0/G1: apoptotic fraction. Values are each the mean ± S.E.M. of three independent experiments, each performed in triplicate.

to 99% at 15 mM. RES showed proliferative activity at 1 μM because of a higher percentage (23%) of mitotic cells in the G2/M phase in comparison to the control (17%) (Fig. 2a, Table 1). Furthermore, 50 μM RES induced almost equal percentage (17%) of cells in the S phase, concomitantly with a significant decrease in percentage of cells in the G0/G1 (30%) in comparison to the control (47%) and to a much higher decrease in the G2/M-phase (9%) in comparison to the control (17%) (Fig. 2a, Table 1). This shows accumulation of cells in the S phase. Noticeably, higher dose of RES caused a significant decrease in the G0/G1-, S-, and G2/M-phase and induced apoptosis in a drug concentration–dependent manner, reaching values close to 74% at 200 μM (Fig. 2b). Combination doses of CPA and RES decreased the G0/G1 peak, which illustrates a shift towards the sub-G0/G1, indicative of apoptosis (Fig. 2b).

Analysis of apoptosis by annexin V-FITC and PI staining

Fluorophores annexin V and PI were used to analyze progressive stages of apoptosis. Control, untreated cells showed rare presence of annexin V-FITC (faint green fluorescence), indicating the presence of basal, early stage apoptosis in the cells at steady state (Fig. 3). A fair amount of phosphatidylserine (PS) externalization (green fluorescence) on the plasma membrane was noticed upon exposure with drugs beginning at 5 mM CPA and 50 μM RES, indicating the onset of the apoptotic phenomena (Fig. 3). Higher concentrations of CPA (10 mM) and RES (100 μM) showed a high fraction of PI accompanying a moderate amount of annexin V staining, depicting mid-phase apoptosis (Fig. 3). The combination dosage of CPA and RES, that is, 5 mM CPA with 50 μM RES, showed a fair annexin-positive fraction and a strong PI-positive fraction that was further raised to intense red staining of the nuclear region with no sign of green stain at 10 mM CPA with 100 μM RES in MCF-7 cells, depicting the late stage of apoptosis (Fig. 3). This provided us a lead to use the ideal combination of 5 mM CPA with 50 μM RES for further studies.

Assessment of activities of caspases

In MCF-7 cells, caspase-3 activity was found to be negligible at all the doses of drug alone or in combination, suggesting the absence of functional caspase-3 (11). The activities of caspase-8 and -9 increased in a dose-dependent manner with either ligand (5 and 10 mM CPA, 50 and 100 μMRES) or their combination (5 mM CPA plus 50 μM RES, 10 mM CPA plus 100 μM RES). In MCF-7 cells, the combination is comparatively more efficient than drugs alone in significantly inducing caspase-8 and caspase-9 (Fig. 4).

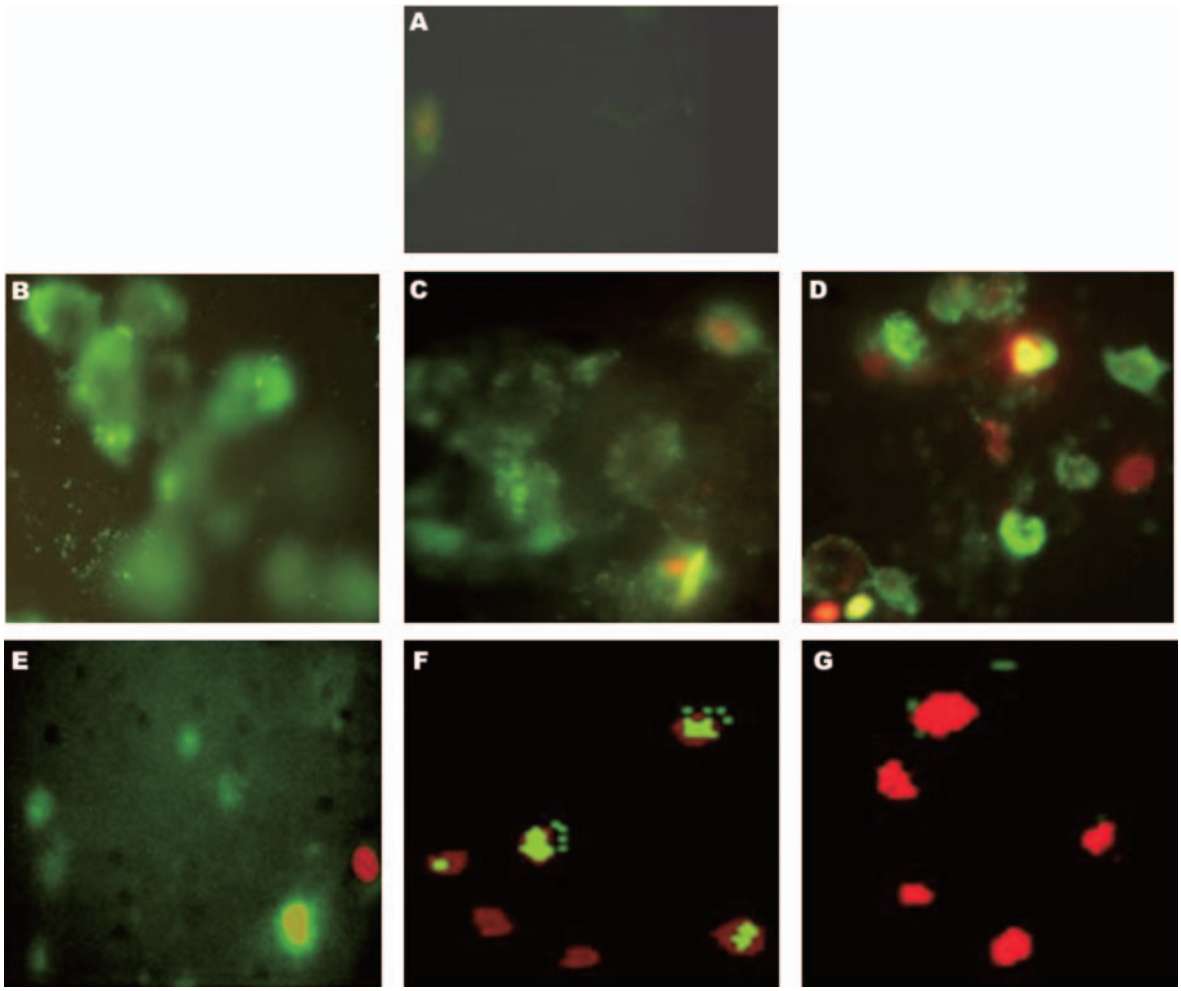


Fig. 3. Detection of apoptosis in MCF-7 cells by annexin-V-FITC and PI staining. Briefly, 0.4×10^6 cells were pre-cultured for 48 h in phenol red–free DMEM (DCC/FCS-treated) and treated with different doses of CPA [5 (B) and 10 mM (E)], RES [50 (C) and 100 μM (F)], and CPA plus RES [5 mM CPA + 50 μM RES (D) and 10 mM CPA + 100 μM RES (G)] for 48 h. After culmination of incubation with drug, cells were stained with annexin V-FITC (green fluorescence) and PI (red fluorescence) and analyzed by fluorescence microscopy (magnification 400×).

Effects of caspase inhibitor on apoptosis

The pan-caspase inhibitor Z-VAD-FMK prevented the occurrence of CPA-, RES-, and CPA plus RES–induced apoptosis of MCF-7 cells (Fig. 5). Inhibition of caspases was also seen in control cells because starvation of cells by growing them in DCC/FCS media can effectively induce apoptosis in rapidly growing cells in vitro (Fig. 5).

Phospho-Ser15-p53, Bax, Fas, $p21^{WAF/Cip1}$, and Bcl-2 expression in drug-treated MCF-7 cells

Treatment of CPA, with and without RES, leads to rapid increase of phospho-Ser15-p53 cellular levels (Fig. 6: a and c) as compared to control MCF-7 (possessing functional wild-type p53: 12, 13), while the combination showed maximum increase of phospho-Ser15-p53 up to 28-fold (Fig. 6c). Since, p53 up regulates Bax, Fas, and $p21^{WAF/Cip1}$ and down regulates Bcl-2 (14 – 18), CPA with and without RES-treated cells showed up-regulation of $p21^{WAF/Cip1}$ levels, Fas, and Bax protein as compared to the untreated control cells. There was no alteration in the expression of Bcl-2 and Bax in CPA-treated cells (Fig. 6: b and c), but RES induced a marked increase in pro-apoptotic Bax protein (1.22-fold increase) and a significant decrease ($P = 0.01$) in anti-apoptotic bcl-2 (2.57-fold decrease) in MCF-7 cells (Fig. 6: b and c). On the other hand, expression of Bcl-2 and Bax decreased and increased up to 5-fold respectively in CPA plus RES–treated cells as compared to the control (Fig. 6: b and c). The combination of CPA with RES showed enhanced cytotoxicity compared to the individual drugs alone through maximum levels of membrane bound FasL and Bax and minimum levels of Bcl-2 (Fig. 6: b and c). Interestingly, RES decreased the

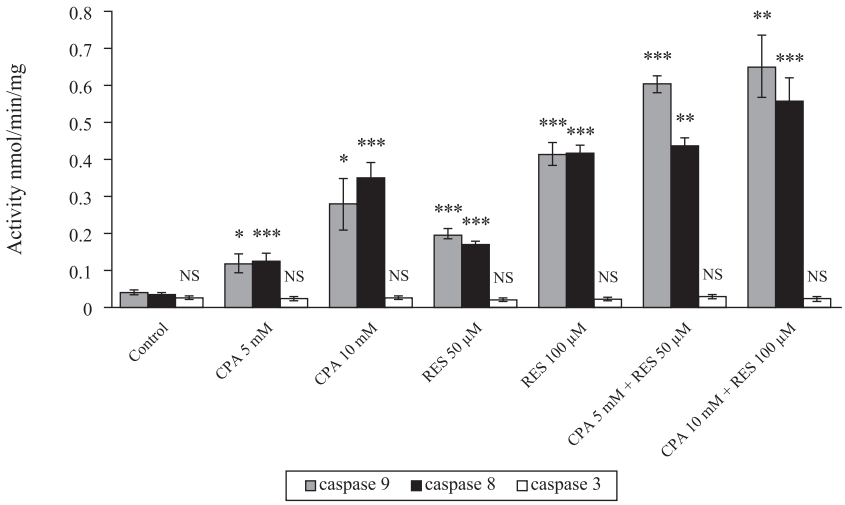


Fig. 4. Assessment of caspase activities in CPA-, RES-, and CPA plus RES–induced apoptosis in MCF-7 cells. Cells were pre-cultured for 48 h in phenol red–free DMEM (DCC/FCS-treated) and subsequently exposed with different doses of CPA (5 and 10 mM), RES (50 and 100 μM), and CPA plus RES (5 mM CPA + 50 μM RES and 10 mM CPA + 100 μM RES) for 48 h. Cell lysates were prepared and treated with caspase-8 substrate (Z-IETD-pNA), caspase-9 substrate (Ac-LEHD-pNA), and caspase-3 substrate (Z-DEVD-pNA). Activities were measured as a function of pNA released. Bars: Mean ± S.E.M. *P<0.05, **P<0.01, ***P<0.001 (CPA /RES/CPA plus RES–treated cells as compared to the control).

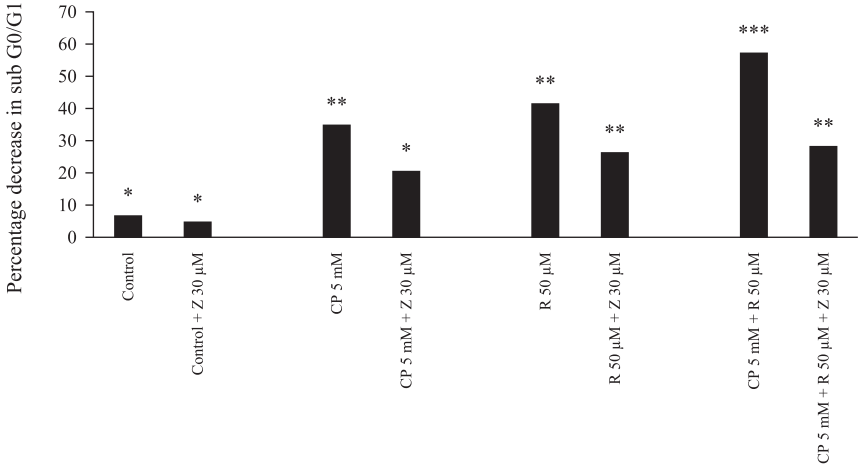


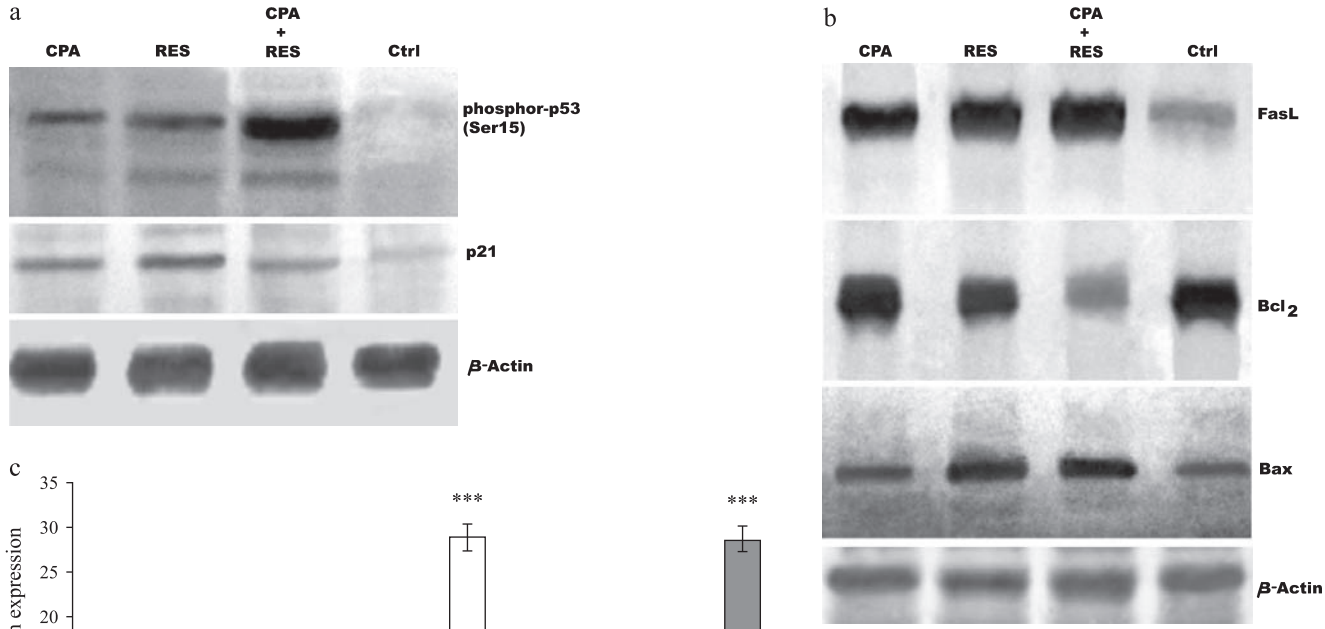
Fig. 5. Determination of the inhibition of CPA-, RES-, and CPA + RES–induced apoptosis of MCF-7 cells in a co-culture with the pan-caspase inhibitor Z-VAD-FMK. MCF-7 cells (0.5×10^6) were preincubated with Z-VAD-FMK (30 μM) for 1 h prior to the addition of 5 mM of CPA, 50 μM of RES alone, and 5 mM CPA + 50 μM RES for 48 h in phenol red–free DMEM (DCC/FCS-treated). The cells were harvested, permeabilized, stained with PI (40 μg/ml), and analyzed by FACS (Cell Quest Software). Each data value is the mean of three similar experiments, each performed in triplicate. *P<0.05, **P<0.01, ***P<0.001 (CPA /RES/CPA plus RES–treated cells as compared to the control).

CPA-mediated increase in $p21^{WAF/Cip1}$ levels (Fig. 6: a and c). The evidence presented here suggests that p21 may modulate apoptosis induction in MCF-7 cells.

Discussion

We propose a detailed mechanism for the CPA- and RES-induced apoptosis in human breast cancer cells that may explain combination-induced breast cancer regression in ER + MCF-7 cells. CPA with and without RES showed cytotoxic effects in the cultured human breast cancer cells (HBCCs) MCF-7 and MDA-MB-231, while HEK 293, MDCK, and Hep G2 cells showed no sensitivity (Fig. 1: e – g). The disparity of action of drugs towards normal (HEK 293, MDCK) versus cancerous cells (Hep G2, MCF-7, and MDA-MB-231) may be attributed to absence of differences in tissue-specific levels and expression patterns of cytochrome P450 (CYP) isoforms (19). However, although it has been reported that CYPs are expressed in normal tissue, they are expressed at much higher level in cancerous cells and are lost during in vitro cultivation (20). Moreover, cytotoxicity in HBCCs cells may also be due to a significant amount of NADPH cytochrome P450 reductase, which transfers electrons required for all microsomal cytochrome P-450–dependent enzymatic reactions (21). Subsequently annexin V assay, in conjunction with the vital dye PI, a sensitive method for the detection of progressive stages of apoptosis (22), supported the contentions that doses of both drugs when used in combination could safely be reduced without compromising therapeutic efficacy (Fig. 3).

Flow cytometry analysis using PI staining, confirmed apoptosis as a cell death mechanism in the MCF-7 cell line. The G1 arrest at the initial concentrations of CPA (1 – 2.5 mM) portray cell-cycle arrest so that cells may be provided with enough time to repair damages, but at higher concentrations, the damage increases to such a



	CPA	RES	CPA + RES
Bcl-2	1.072202486	2.570342119	5.441171578
Bax	1.061332877	1.221837311	4.907614854
FasL	4.072901957	4.290599448	9.342023148
p21	5.506992548	28.93362237	2.975053975
p53	13.49709913	7.379444716	28.70909452

Fig. 6. Evaluation of expression of phosphor-p53 and its target gene $p21^{WAF/Cip1}$ in cells treated with CPA, RES, and CPA plus RES. Briefly, 4×10^6 cells were pre-cultured for 48 h in phenol red–free DMEM (DCC/FCS-treated) and subsequently treated with different doses of CPA (5 mM), RES (50 μM), and CPA plus RES (5 mM CPA + 50 μM RES) for 48 h. Standard western blot analysis was performed using antibodies against phospho-p53 (Ser15), $p21WAF/Cip1^{WAF/Cip1}$ (a); Fas, Bcl-2, Bax (b). Loading conditions were evaluated by probing the same membrane with anti-β actin antibody. c: densitometric quantitation of protein expression levels are shown as fold changes. *P<0.05, **P<0.01, ***P<0.001 (CPA/RES/CPA plus RES–treated cells as compared to the control). Each data value is representative of three identical experiments.

high extent that the arrest is overcome by cell death or apoptosis (Fig. 2a). The amply documented anticancer properties of RES are related to its abilities to cause cell cycle arrest in the G1 phase (23) or in the S-G2 phase transition (24 – 27), and it triggers apoptosis in a variety of cancer cell lines (28 – 31). RES induces specific biochemical effects in cell culture models in the 1 – 10 μM range, and its cytostatic and cytotoxic effects usually require 25 – 100 and 100 – 200 μM concentrations, respectively. However in our study, RES in MCF-7 cells caused S phase arrest at 1 – 50 μM, but at higher concentrations of RES (100 – 200 μM), there was dose-dependent increase in apoptosis (Fig. 2a). The combination of CPA with RES in MCF-7 cells released the arrest of the G1 or S phase and increased the sub G0/G1 phase, leading to the best possible apoptotic response, which may be due to increased DNA damage (Fig. 2b). The DNA laddering characteristic of apoptosis (data not shown) in MCF-7 cells occurred in a dose- and time-dependent manner, reconfirming that CPA with or without RES induced apoptosis. In response to DNA damage to the cell, the posttranslational modification of tumor suppressor protein p53 is known to inhibit cell growth and apoptosis (32). The p53 gene is also known to regulate the G1 checkpoint, which can either induce cell cycle arrest (increased expression of p21 Waf1/Cip1) or initiate apoptosis (decreased expression of p21 Waf1/Cip1). A link between decreased levels of p21 Waf1/Cip1 and an increased tendency toward apoptosis has also been reported in prostrate cancer cells and a

growth factor–dependent murine hematopoietic cell line (33, 34). CPA and RES alone increase the p21 Waf1/Cip1 expression, which is suggestive of cell-cycle arrest; and further down-regulation of p21 Waf1/Cip1 in combination renders MCF-cells more susceptible to the apoptotic stimuli (Fig. 6: a and c). Furthermore, the increased level of expression of stress activated serine phosphorylation of p53 tumor suppressor observed with the drug combination as compared to the drugs alone further support the best apoptotic response (Fig. 6: a and c). To summarize, our present observations show that over-expression of p53 in combination-treated MCF-7 cancer cells can overcome a stable cell cycle arrest (MCF-7 cells treated with either CPA or RES alone), resulting in an enhanced apoptotic cell death (35).

Furthermore, pan-caspase inhibitor Z-VAD-FMK was used to delineate the detailed mechanism of CPA with and without RES-induced apoptosis. Our results using Z-VAD-FMK a cell-permeable pan-caspase inhibitor demonstrate that CPA with and without RES-induced apoptosis depends on specific caspases (Fig. 5). Caspase-8 plays a critical role in the early cascade of apoptosis, acting as an initiator of the caspase activation cascade (36). The early apoptotic initiator caspase-8 increased in a dose–dependent manner in our study with either ligand alone (5 and 10 mM CPA, 50 and 100 μM RES) or with their combination (Fig. 4). The intrinsic or mitochondrial pathway releases pro-apoptotic factors such as cytochrome *c* and apoptosis inducing factor (AIF), which then initiates downstream caspases such as caspase-9 and -3 to execute the apoptotic events (37, 38). We detected increased caspase-9 activity in a dose-dependent manner with either ligand (loc cit) or their combination (Fig. 4). Hence the combination was comparatively more efficient in generating early apoptotic initiator caspase-8 and caspase-9 than CPA or RES alone, which suggests the involvement of both extrinsic and intrinsic pathways. We detected no caspase-3 activity, which is known to be absent in MCF-7 cells due to the functional deletion of the *CASP-3* gene (11). However, caspase-3 like proteases are operative in these cells since they have been reported to undergo caspase-dependent cell death (39).

To further delineate the upstream events, we analyzed CPA (with or without RES)-induced regulation of extrinsic and intrinsic pathways of apoptosis. The control MCF-7 cells (grown in DCC/FCS to deplete the cells of steroids) show an ample amount of Bcl-2 expression, resulting in the inhibition of apoptosis in MCF-7 cells (Fig. 6b). CPA alone showed no changes in Bax/Bcl-2 ratio, on the other hand; a marked increase in pro-apoptotic Bax protein and a decrease in anti-apoptotic Bcl-2 were induced by RES in MCF-7 cells (Fig. 6: b and c). In RES-treated MCF-7 cells, a significant decrease in Bcl-2 was noted without same level of changes in Bax. However, the activation of the intrinsic apoptotic pathway is correlated with changes in the ratio of both proteins rather than with individual variations (24). Therefore, our results suggest that RES induced apoptosis in MCF-7 cancer cells through alteration of the Bax/Bcl-2 balance. Furthermore, the combination augments the Bax:Bcl2 ratio to much higher extent as compared to the drugs alone. Hence, increased levels of Bax and decreased levels of Bcl-2 correlates with the good in vitro response of combination chemotherapy. The results of members of the Bcl-2 family of proteins and p53 are in agreement with the observations in CPA- and RES-induced apoptosis in breast carcinoma cells, where levels of these proteins were changed (4, 6).

Furthermore, a number of studies have suggested that anticancer drugs kill susceptible breast cancer cells by inducing the expression of death receptor ligands such as Fas Ligand (Fas L). The Fas expression in variety of different cell lines depends on the presence of wt p53 gene (40). MCF-7 cells have functional wild-type p53 (12); hence, Fas L involvement is suggested in CPA (with or without RES)-treated cells. FasL (CD95L, Apo-1L) exists in two forms, the soluble form and membrane-bound form (40). Soluble FasL is less potent than the membrane-bound form, and cell surface "shedding" of the ligand is the mechanism for the inactivation of its cytotoxicity. Therefore, we have not used conditioned media for detecting soluble FasL. Our results demonstrate the combination of CPA with RES enhanced apoptosis through the death-receptor pathway by increasing the levels of membrane bound Fas L in comparison to the individual drugs alone (Fig. 6: b and c). Thus, these findings are the first to show that both the mitochondrial and Fas L system plays an important role in CPA plus RES–mediated MCF-7 cellular apoptosis.

RES ($\geq$50 μM) is effective in both ER + (MCF-7 cells) and ER-ve (MDA-MB-231) breast cancer cell lines as reported by us as well as in earlier studies (24). Besides this, it has been proven that under the in vivo condition, metabolites of RES play major roles in chemopreventive activity (41, 42). CPA, which causes auto-induction of CYPs (43) when combined with RES, may also enhance the metabolism of RES; and the combination of CPA plus RES may provide better efficacy under in vivo conditions. The in vivo toxicity of RES is not a major issue since very few reports are available about this. Recently, it has been reported only high doses of RES (3 g/kg per day) cause moderate liver toxicity (44). This potentially toxic concentration is much higher than the dose of RES used in this study. So RES may not be toxic under physiological conditions when used

at the doses employed in the present study. Moreover, due to its poly-phenolic nature, metabolites of RES could conceivably accumulate in fatty tumor tissue, which is abundantly present in the breast. Therefore, even in lower doses, it may provide enough metabolite for its anti-neoplastic activity in the breast.

Acknowledgments

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