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**Induction of Apoptosis in Breast Cancer Cells By *Saccharomyces
Cerevisiae*, The Baker's Yeast, *In Vitro***

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Ghoneum and Gollapudi: Yeast-induced Apoptosis of Cancer Cells

Key words: Phagocytosis, apoptosis, breast, cancer cell, *in vitro*.

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Abstract.

The present study was undertaken to evaluate the effect of phagocytosis of killed yeast on the induction of apoptosis in human metastatic breast cancer cells (MCF-7 and ZR-75-1) and non metastatic breast cancer cells (HCC70). Heat-killed *Saccharomyces cerevisiae*, baker's and brewer's yeast, was cultured with cancer cells at a ratio of yeast to cancer cells = 10:1, and the percent apoptotic cancer cells was determined by flow cytometry and cytospin preparation. Upon phagocytosis of yeast, breast cancer cells underwent apoptosis. Induction of apoptosis was time- and dose- dependent. Apoptosis was detected as early as 0.5 h (13%), increased to 19% at 2 h and peaked (38%) at 4 h. Metastatic cancer cells were found to be more susceptible to yeast-induced apoptosis than non metastatic cells; 629% increase for MCF-7 as compared to cells alone, 258% for ZR-75 cells, while HCC70 cells showed a 178% increase. Phagocytosis is associated with the disruption of mitochondrial membrane potential and activation of initiator and effector caspases 8, 9 or 3. However, inhibitors of these caspases did not inhibit yeast-induced apoptosis in cancer cells, suggesting that yeast-induces apoptosis in breast cancer cells by a mechanism that is independent of caspase activation. This data may have clinical implication.

Apoptosis constitutes a strictly programmed device for the removal of aged, damaged and abnormal cells. Apoptosis can be triggered by a diverse number of stimuli such as exposure to chemical and physical agents (1), oxidative stress (2) and the removal of growth factors (3). Many anticancer drugs function by inducing apoptosis (4-10). Research in the last decade revealed a promising future for apoptosis-based cancer therapies. The specific intercellular damage induced by many therapeutic agents has been characterized and shown to involve Fas/FasL system (7), mitochondria (8) and DNA damage (9, 10). However, the chemotherapeutic agents are known to exhibit an indiscriminate killing that involves cancer cells and many normal cells. It is therefore of particular interest to find agents that induce apoptosis of cancer cells, but with minimal side-effects. In this study we demonstrated that phagocytosis of non-pathogenic yeast, *S. cerevisiae* induces apoptosis in breast cancer cells (BCCs).

The phenomenon of induction of apoptosis after phagocytosis of certain microorganisms has been well studied in the human phagocytic cells. Several studies indicated an increase in the expression of apoptosis by neutrophils and monocytes/macrophages post phagocytosis of microorganisms such as *Escherichia coli* (11), *Mycobacterium tuberculosis* (12), *Staphylococcus aureus* (13), *Candida albicans* (14) and *Shigella flexneri* (15). Earlier studies demonstrated that BCCs in culture can phagocytise latex beads and fluorescent Matrigel (16, 17). We have recently confirmed the phagocytic activity by BCCs using yeast as a test organism (18). The results of this study demonstrated that phagocytosis of heat-killed *S. cerevisiae* induces apoptosis in both highly metastatic cells (MCF-7 and ZR-75-1) and non-metastatic cells (HCC70) in a caspase-independent mechanism. This observation may have therapeutic implication.

Materials and Methods

Tumor cell lines. Four human tumor cell lines were used in the present study. These were human metastatic breast cancer cell lines (MCF-7 and ZR-75-1) and a human non-metastatic breast cancer cell line (HCC70). In addition, a human macrophage cell line (U973) was used. All cell lines were purchased from American Tissue and Culture Collection (ATCC), Manassas, VA, U.S.A. Tumor cells were maintained in our laboratory in RPMI-1640, supplemented with 10% fetal calf serum (FCS), 2 mM glutamine and 100 µg/ml of streptomycin and penicillin.

Preparation of *S. cerevisiae*. Commercially available baker's and brewer's yeast, *S. cerevisiae*, was used in suspensions that were washed once with phosphate-buffered saline (PBS). It was then incubated for 1 h at 90°C to kill the yeast and washed 3 times. Quantification was carried out using a hemocytometer and cell suspensions were adjusted to 1×10^7 cells/ml.

Phagocytic assay. A previously reported phagocytic assay was employed with slight modifications (19, 20). In brief, tumor cells were mixed with yeast at a ratio of yeast to tumor cell of 10:1. For this purpose, a 0.5 ml tumor cell suspension in culture medium containing 1×10^6 cells/ml was mixed with 0.5 ml yeast suspension containing 1×10^7 organisms/ml. The mixtures were centrifuged in capped plastic tubes (16 x 100 mm; Falcon Plastic, Los Angeles CA, USA) for 5 min at 50 xg and incubated at 37 °C and 5% CO₂. After 0.5, 2 and 4 h incubation, the mixtures were thoroughly resuspended to detach loosely attached yeast from tumor cells. Cell suspensions (200 µl) were used to make cytospin preparations (Shandon Southern Instruments, Sewickly PA, U.S.A). Preparations were fixed in 100% methanol, air-

dried, stained with 4% Giemsa for 15 min. (Sigma –Aldrich Corp., St. Louis, MO, U.S.A) and examined using oil immersion and a light microscope fitted with 100x objective (Nikon, Tokyo, Japan).

Apoptosis studies

(A) *Detection of Breast cancer cell (BCCs) viability using flow cytometry:* Flow cytometry analysis was used to examine the percentage of dead cancer cells. BCCs were cultured in the presence or absence of yeast cells at a ratio of 1:10 and the percentage of dead cancer cells was examined by propidium iodide technique. Briefly propidium iodide (PI) was added to cells (1×10^6 /ml) to give a final PI concentration of (5 μ g/ml) Cells were stained for 30 min at room temperature in the dark and analyzed by FACScan (Becton Dickinson, San Jose, CA, U.S.A).

(B) *detection of apoptic cancer cells by morphological analysis:* Apoptosis is morphologically defined by cell shrinkage, membrane blebbing and chromatin condensation. These criteria were used to identify the apoptic cancer cells in cytospin preparations stained with Giemsa. A separate set of experiments of phagocytosis was carried out to investigate apoptosis of BCCs at different ratios of cancer cells to yeast = 1:2, 1:5, 1:10 and 1:25.

(C) *Detection of mitochondrial potential $\Delta\psi_m$:* Variations of the mitochondrial transmembrane potential $\Delta\psi_m$ during apoptosis was studied using 3'3'-dihexyloxacarbocynine dye (DIOC₆(3)) (Molecular Probes, Eugene, OR, U.S.A). Briefly, 5×10^5 cells/ml were incubated with 0.5 μ M DIOC₆(3) for 30 min at 37 °C. Cells were transferred on ice for FACS analysis. Forward and side scatters were used to gate and exclude cellular debris using a FACScan. Cells were excited

at 488 nm and green was collected on FL1 at 530 nm. Five thousand cells were analyzed. Data were acquired and analyzed using Cell Quest software (Becton-Dickinson).

(D) Determination of activation of caspases 8, 9 and 3: The method is based on carboxyfluorescein-labeled fluoromethyl ketone (FMK)-peptide inhibitors of caspases. These inhibitors are cell permeable and non-toxic. Once inside the cells, these inhibitors bind covalently to the active caspase. Caspase positive (+) cells are distinguished from caspase negative (-) cells with aid of flow cytometry. Briefly, cells undergoing apoptosis were loaded with fluorescein-labeled FMK-peptide inhibitors (FAM-LETD-FMK for caspase 8, FAM-LEHD-FMK for caspase 9, FAM-DEVD-FMK for caspase 3, Intergen Company, NY, U.S.A). After 1 h incubation, the cells were washed to remove unbound caspase and cells that contain bound inhibitor were quantified using a FACScan flow cytometer.

(E) Caspase inhibitors: To study a role of caspases in apoptosis, 1×10^6 cells of BCCs (MCF-7 and ZR-75) were cultured with caspase inhibitors at two different concentrations (1 and 4 μM) of caspase inhibitors (Z-DEVD-FMK, Z_LETD-FMK and Z-LEHD-FMK, BioVision, Palo Alto, CA, U.S.A). At 30 min, cells were washed twice with HBSS and cultured with yeast at the ratio of 10:1 for 2 h. The percentage of dead cancer cells was examined by flow cytometry as described above.

Statistical analysis. Using the Student's *t*-test, we tested the significance of difference in the percent changes of apoptic cancer cells post culture with yeast as compared to cancer cells alone.

Results

Three BCC lines (MCF-7, ZR-75, and HCC-70 cells) were cultured with *S. cerevisiae* and the percent of apoptosis and activation of caspases 8, 9, 3 were examined.

Morphological identification of apoptic BCCs by cytospin preparations. Apoptosis is morphologically defined by membrane blebbing and chromatin condensation. These criteria were used to identify the apoptic BCCs in cytospin preparations. Apoptosis occurred post phagocytosis of yeast by BCCs. At 1 h post culture yeast with cancer cells, MCF-7 cells exhibited increased phagocytic activity against yeast (Fig. 1a). This was followed by the gradual demise of cancer cells starting with early chromatin condensation (Figs. 1b & c). Notice that the nucleus occupies about half of the cell. Subsequently the chromatin condensation was further intensified (Fig. 1d). In addition, cancer cells with membrane blebbing were clearly identified during yeast-induced apoptosis (Figs. 1e & f). This was followed by enlargement of the cancer cell (Fig. 1g). Notice yeast inside apoptic MCF-7 cells. Finally, the nucleus disappeared and the cell disintegrated (Fig. 1 h).

Standardizing experiments of apoptosis

1. Different ratios of MCF-7 cells to yeast: MCF-7 cells were co-cultured with yeast at different ratios: 1:2, 1:5, 1:10 and 1:25. At 2 h, flow cytometry analysis was used to examine the percentage of dead cancer cells. Fig. 2 shows that the low ratio of yeast to cancer cells at 2:1 induced 5.3% of dead cancer cells; this was increased to 9.2% at the higher ratio of 5:1. The increase of percentages became significant at ratios of 10:1 and 25:1, and showed 21.4% and 30.3% apoptic MCF-7 cells, as compared with the control untreated cells (5%).

2. Time intervals of co-culture MCF-7 cells with yeast: The percentage of apoptosis was examined in cytopsin preparations. Data in Fig. 3 shows that MCF-7 alone revealed 5% cells in apoptosis. Treatment with yeast induced apoptosis of cancer cells; its percentages increased with an increase in time of incubation with yeast. It was first detected at 0.5 h (13%); at 2 h it increased to 19% and maximized to 38% at 4 h.

3. Determination of percentages of dead BCCs post treatment with yeast by flow cytometry:

BCCs were cultured with yeast at a ratio of 1:10 for 2 h and cancer cell survival was determined by flow cytometry using propidium iodide (PI) technique. In this technique dead cells pick up PI and fluoresce. The results depicted in Fig. 4 show that the background of dead BCCs ranged between 3.4 – 6.6%. Co-culture of cancer cells with yeast resulted in significant decrease in BCCs survival: 636% for MCF -7 cells and 260% for ZR-75 cells, while HCC70 showed 180% as compared with the background of cancer cells.

Studies of caspases 8, 9, and 3

1. Yeast induces activation of caspases: Apoptosis of MCF-7 cells is associated by activation of the caspase cascade. In order to determine the steps in yeast-mediated apoptosis, we examined the activation of proximal caspases (caspase 8 and caspase 9). MCF-7 cells were co-cultured with yeast. The proportion of cells with active caspase 8 and caspase 9 was determined with a Caspatag caspase detection kit, using FACScan. The data in Fig. 5 shows that yeast induced the activation of caspase 8 and caspase 9 in MCF-7 cells. For caspase 8, the percentage increased to 18.94% as compared to 3.5% for MCF-7 cells alone; this represents a 541% increase (Fig. 5a). As shown in Fig. 5b, co-culture of yeast with MCF-7 cells resulted in an increase of

caspase 9 activation to 20.4%, as compared to 4.5% for MCF-7 alone; this represents a 453% increase.

A similar trend of activation of caspases post-treatment with yeast was noticed with ZR-75 cells. Data in Figs. 6a-c demonstrated that treatment with yeast increased activation of caspases: 22.6%, 18.5% and 13.1% for caspases 8,9 and 3 respectively as compared to 7.2%, 8.4% and 8.2% for ZR-75 cells alone. HCC70 cells treated with yeast also showed activation of caspases: 20% for caspase 8, 11.6% for caspase 9 and 15.3% for caspase 3 when compared to control untreated cells (Fig. 6a-c).

2. Apoptosis in the presence of caspase inhibitors: Table I shows that treatment of MCF-7 cells with inhibitors of caspases 8 and 9 failed to inhibit yeast-induced apoptosis in MCF-7 cells. Inhibitors at low as well as high doses caused slight fluctuations in the percentage of apoptosis of cancer cells as determined by flow cytometry. Similarly, treatment of ZR-75 cells with inhibitors of caspases 8, 9 and 3 demonstrated no significant differences in the level of apoptosis in cancer cells, as compared to cells treated with yeast in the absence of caspase inhibitors.

Phagocytosis of yeast causes disruption of mitochondrial membrane potential.

Tumor cells (MCF-7 and HCC70) were cultured with yeast for 30 min and the mitochondrial membrane potential was determined by FACScan using DIOC6. Fig. 7a shows MCF-7 cells treated with yeast revealed a significant decrease in the mitochondrial polarization, while HCC70 cells did not demonstrate a significant decrease (Fig. 7b).

Discussion

Phagocytosis by cancer cells is an established phenomenon which was originally identified with the histological examination of human tumors that revealed the presence of intracytoplasmic leukocytes, erythrocytes and blood platelets in tumor cells (21-27). Further studies demonstrated phagocytosis in non-lymphatic tumor cells such as dermatofibroma cells, adenocarcinoma cells, and epithelial carcinomas of the breast (26- 29). Studies in cervical cancer patients and *in vitro* showed malignant cells that do not come from phagocytic origin can exhibit phagocytic activity against autologous lymphocytes (30,31), aged lymphocytes, bacteria and *C. albicans* (20, 32-34). Earlier studies also demonstrated that BCCs in culture can phagocytize latex beads and fluorescent Matrigel (16, 17). We recently confirmed the phagocytic activity of BCCs using yeast as the test organism (18); we also noted that yeast may trigger apoptosis of BCCs. The increase in apoptosis of cancer cells post-phagocytizing yeast was cancer cell line-dependent: MCF-7 > ZR-75 > HCC70 cells. This was examined in cytospin preparation and by flow cytometry.

The diversity of actions of yeast on cancer has been the focus of research by many investigators. Earlier studies by us and others showed that *Candida albicans* act as a biological response modifier, causing an augmentation of murine NK cell activity to kill Maloney Leukemia virus-induced T-cell lymphoma (YAC-1) (35,36). In addition, ergosterol, present in baker's and brewer's yeast, was found to inhibit the growth of human BCCs (MCF-7 and MDA-231 cells) *in vitro* by a mechanism that might involve oxidation products of ergosterol (37). Further studies also showed yeast enzymes can be used in the enzyme/prodrug gene therapy. Zhang *et al.* (38) examined an enhanced human carcinoembryonic antigen (CEA) promoter for yeast cytosine deaminase (γ CD), which converts 5-fluorocytosine to 5-fluorouracil to increase

targeting against intrahepatic colon cancer. In the present study, we demonstrated an additional characteristic of the anticancer activity of yeast which is that phagocytosis of the yeast *S. cerevisiae* induced apoptosis of BCCs *in vitro*.

Data of the present study revealed differential response among different BCC lines towards the apoptic effect of *S. cerevisiae*. Metastatic BCCs such as MCF-7 and ZR-75 cells are highly responsive as compared to non-metastatic cancer cells (HCC70). The reason for this phenomenon is not known, but could be attributed to a difference in the uptake of yeast by cancer cells. MCF-7 and ZR-75 cells have been shown to exhibit high phagocytic activity against yeast as compared with HCC70 cells (18). It is therefore of interest to draw an intimate relationship between the phagocytosis of *S. cerevisiae* and apoptosis of cancer cells. MCF-7 and ZR-75 cells showed high percentages of yeast phagocytosis concomitant with an increased level of apoptosis. On the other hand, HCC70 cells showed a low percentage of phagocytosis concomitant with a low level of apoptosis. Since the levels of attachment were similar among the three cancer cell lines, it is suggested that the uptake of yeast by cancer cells, and not the attachment, may trigger apoptosis of cancer cells.

The mechanisms of apoptosis of BCCs post culture with yeast were directed towards investigating the important role of reactive oxygen intermediates (ROI) as well as the role of caspases. With respect to ROI, the O₂ burst which occurs during phagocytosis is known to play an important role in degrading the phagosomes. Several studies have demonstrated the necessity of ROI in triggering apoptosis. This was evidenced by the fact that apoptosis could be blocked post treatment with antioxidants, or by over-expression of antioxidant enzymes (39-41). It is important to note that the O₂ burst may not be necessary for apoptic cell death. For example: Hug *et al.* (42) and Mushel *et al.* (43) reported the occurrence of apoptosis in anaerobic

conditions where ROI are not generated. In addition, induction of an O burst in monocytes by heat-killed bacteria, latex bead and antioxidant N-acetyl-L-cysteine did not trigger apoptosis (14, 44). With respect to BCCs, we have recently shown that the culture of MCF 7 cells with yeast did not result in a respiratory burst (18), suggesting that the engulfed yeast could induce apoptosis by a mechanism independent of ROI.

The precise mechanisms by which yeast induces apoptosis in BCCs need to be investigated. In this study, we showed that phagocytosis of yeast was associated with activation of caspases 8, 9 and 3 in the BCC lines used. Interestingly, the caspase inhibitors failed to block apoptosis in BCCs (MCF-7 and ZR-75 cells). Recently, evidence has also revealed that caspases are not the sole effectors of apoptosis and that induction of cell death can bypass the caspases pathway. van der Kolk *et al.* (45) demonstrated anti-CD20-induced caspase activation in B cells, but the demise of B cells was not inhibited by a broad spectrum of caspase inhibitors Z-VAD-FMK. Similarly, Egger *et al.* (46) showed that Z-VAD-FMK inhibited the activation of caspases in fibroblast cell lines, but not apoptosis in fibroblast cells. The fibroblast cells were triggered to undergo apoptosis with agents that induced endoplasmic reticulum stress. Taken together, our results suggest that *S. cerevisiae* induces apoptosis in BCCs by a caspase-independent mechanism. A number of potential mediators of caspase-independent cell death have recently been identified; these include mitochondrial proteins AIF, an NADH oxidoreductase, endonuclease G, a mitochondrial DNA repair enzyme and HtrA2/Omi, a serine protease (45-51). In response to apoptotic stimuli, these mediators are released from mitochondria and transfer death signals to the nucleus in a caspase-independent manner. In the present study, we showed that phagocytosis led to disruption of mitochondrial membrane potential. It is possible that the release of some or all of the above mentioned mediators might have led to the apoptosis of

BCCs. Experiments are ongoing to identify the exact mediators that induce apoptosis in BCCs upon phagocytosis of *S.cerevisiae*.

In summary, the present study provides the first evidence that heat-killed non pathogenic yeast induce apoptosis of BCCs *in vitro*. The effect is rapid (2 h) and significant (4.3% for MCF-7 alone, extending to 21.9% for cancer cells + yeast). The effect was dose- and time-dependent and operates in a caspase-independent mechanism. This data may establish the foundation for *in vivo* studies that could have therapeutic implications.

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Figure 1. Morphological examination of apoptic MCF-7 cells post phagocytizing yeast. a) Preparation showing increased phagocytic activity against yeast at 1h post culture cancer cells with yeast. b&c) MCF-7 cells showing signs of apoptosis. Notice early chromatin condensation and the nucleus occupying about half of the cell. d) Chromatin further condensed. e&f) Preparations showing cancer cells with membrane blebbing, followed by enlargement of cancer cell (g) and finally the nucleus completely disappears and the cell disintegrates (h). Notice yeast is still inside many of the apoptic tumor cells. Figs.1a-h are Giemsa x 740.

Figure 2. Percent of dead MCF-7 cells at different ratios of cancer cells to yeast as determined by flow cytometry. Tumor cells were cultured in the absence (■) or in the presence of yeast (□) at ratios of 1:2, 1:5, 1:10 and 1:25 for 2 h and cancer cell survival was determine by flow cytometry using propidium iodide (PI) technique. In this technique dead cells pick up PI and fluoresce. Data represents the mean \pm SD of 3 experiments. * $p < 0.001$ as compared to baseline.

Figure 3. Percent of apoptic MCF-7 cells at different intervals. Tumor cells were cultured in the absence (■) or in the presence of yeast (□) at ratios of 1:10. The percentage (%) of apoptic tumor cells was determined at 0.5, 2 and 4 h in cytopsin preparations. Data represent the mean \pm SD of 3 experiments. * $p < 0.001$ as compared to control untreated cells.

Figure 4. Percentage of dead cancer cells post treatment with yeast as determined by flow cytometry. MCF-7 cells, ZR-75 cells and HCC70 cells were cultured with yeast in the absence (■) or in the presence of yeast (□) at a ratio of 1:10 for 2 h and cancer cell survival was determined by flow cytometry.

Figure 5. Increased activation of caspases 8 and 9 of MCF-7 cells post culture with yeast. MCF-7 cells were incubated with yeast and (a) intracellular active caspases 8 and (b) caspase 9

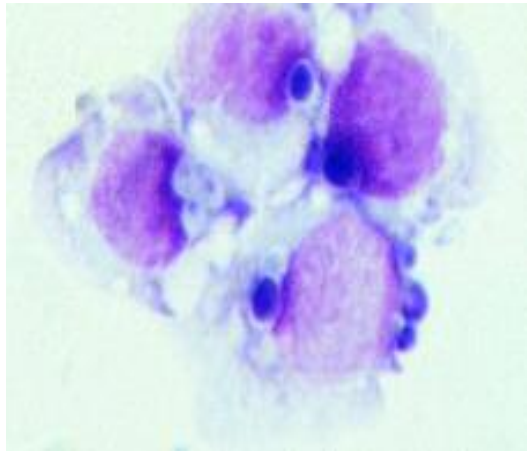
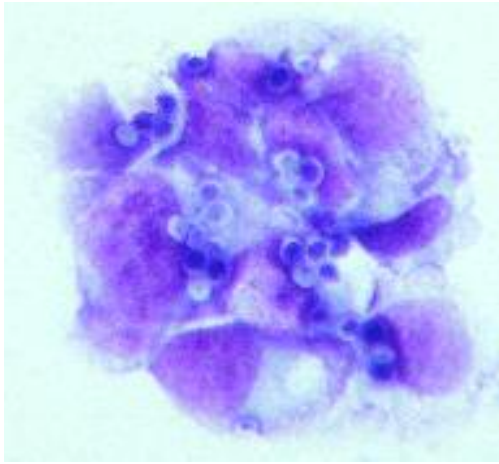
were determined with casp glow caspases 8 determination kit using FACScan. Fig 5 represents a representative dot blot showing increased activation of caspases.

Figure 6. Increased activation of caspases 8, 9 and 3 in three BCC lines post culture with yeast. Cancer cells were incubated without yeast (▨) and with yeast (▩) at 37 °C. After 1 h incubation, the intracellular active caspase 8 (a), caspase 9 (b) and caspase 3 (c) were determined with casp glow caspases 8, 9 and 3 determination kit using FACScan. *Significant at $p < 0.01$.

Figure 7. Effect of MGN-3 on mitochondrial potential. DIOC₆ staining followed by FACS analysis was used to determine the $\Delta\psi_m$ in MCF-7 cells, and MCF-7 plus yeast (a) and HCC70 cells plus yeast (b).

Figure 1a

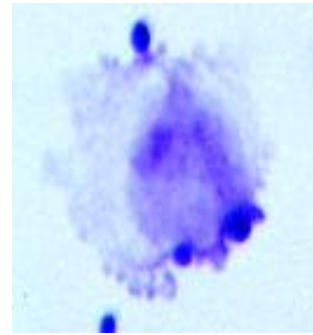
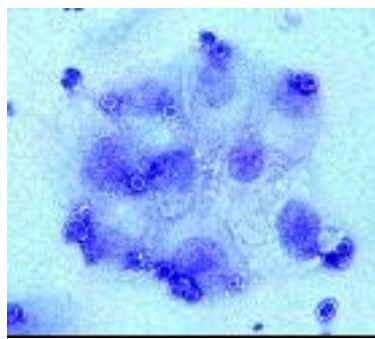
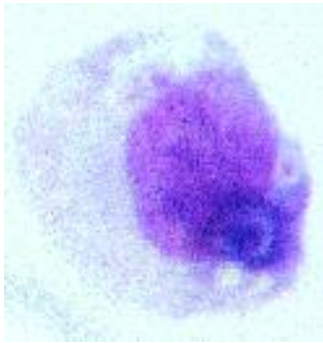
1b



1c

1d

1e



1f

1g

1h

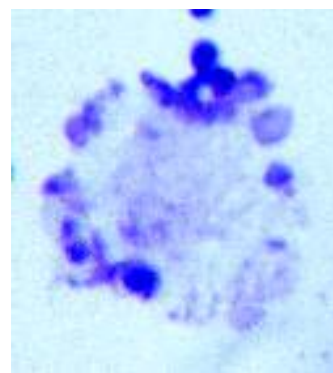
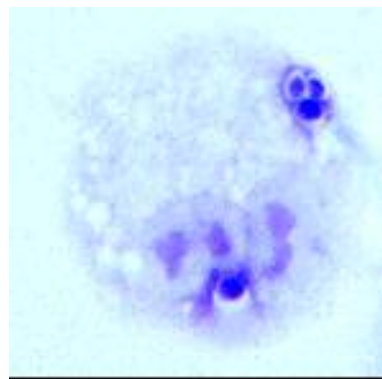
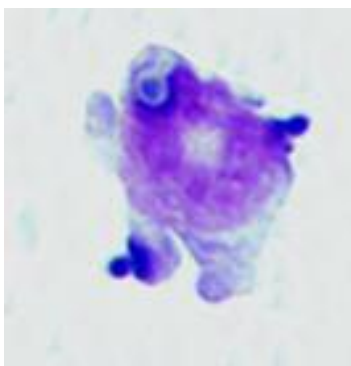


Figure 2

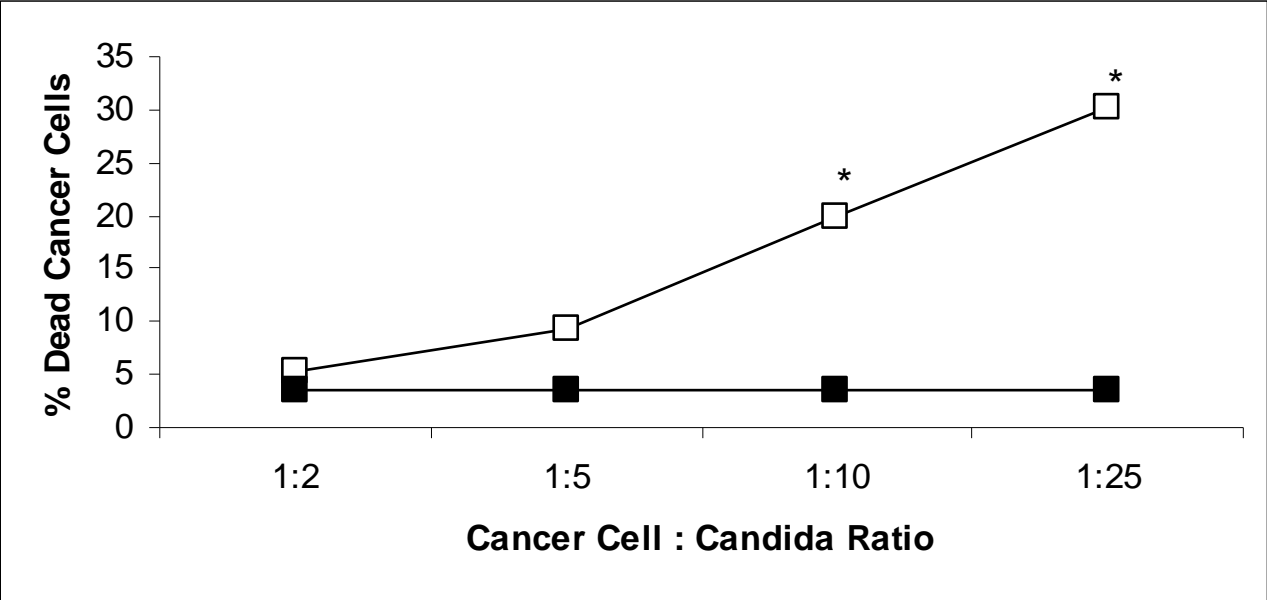


Figure 3

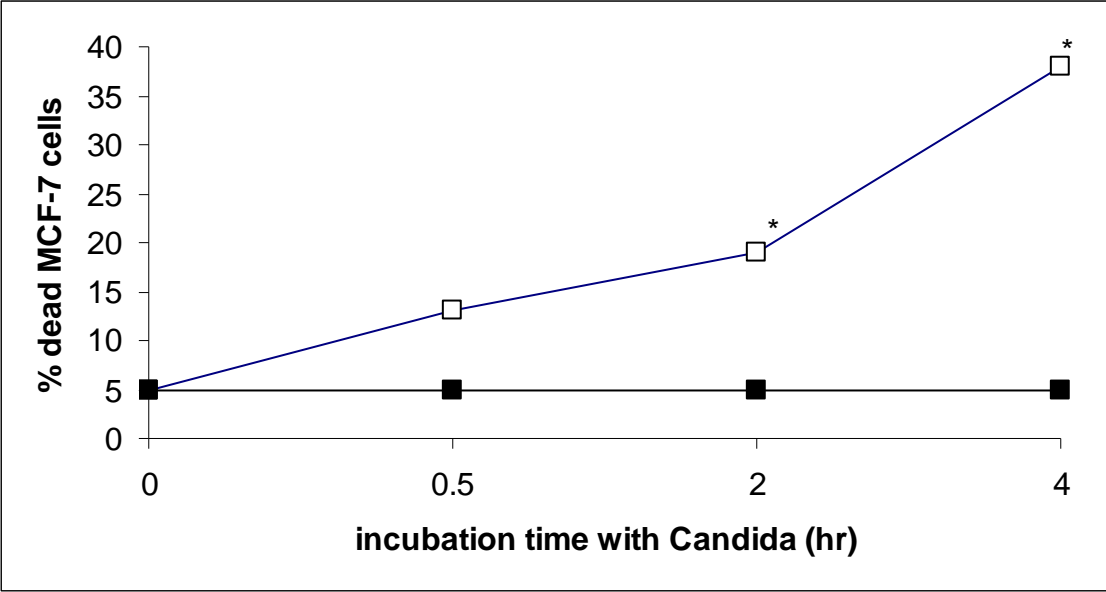


Figure 4

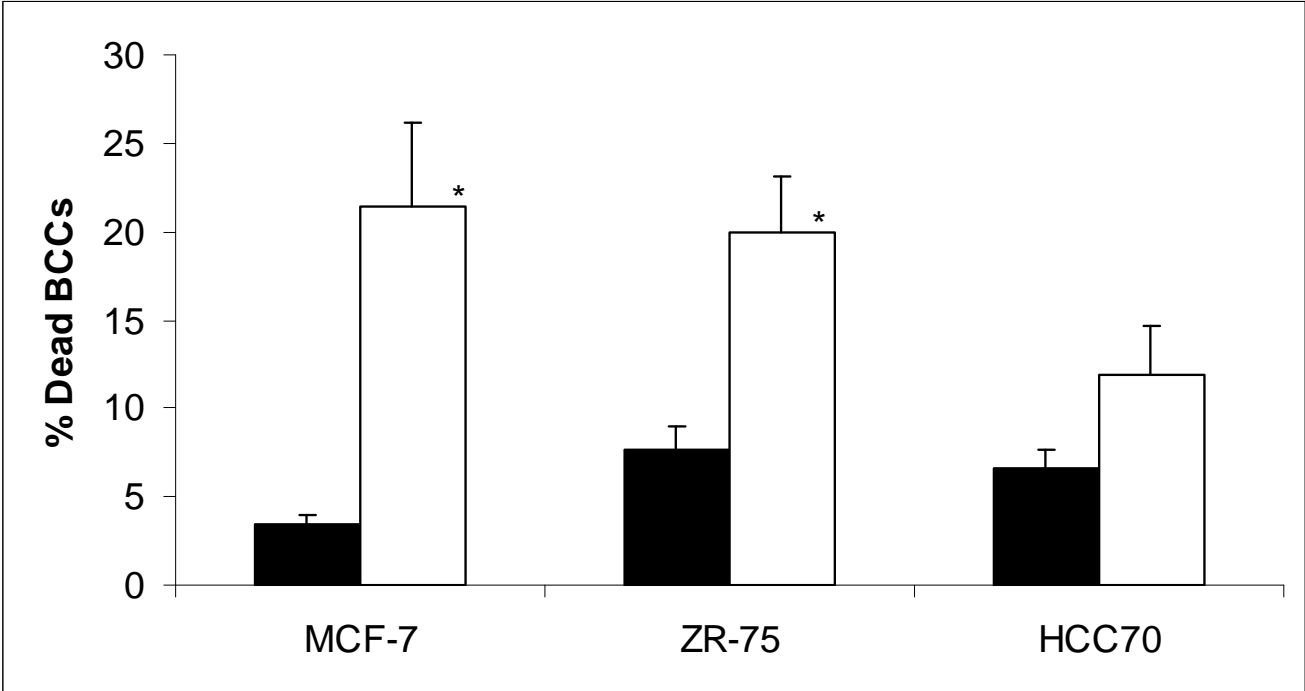


Figure 5

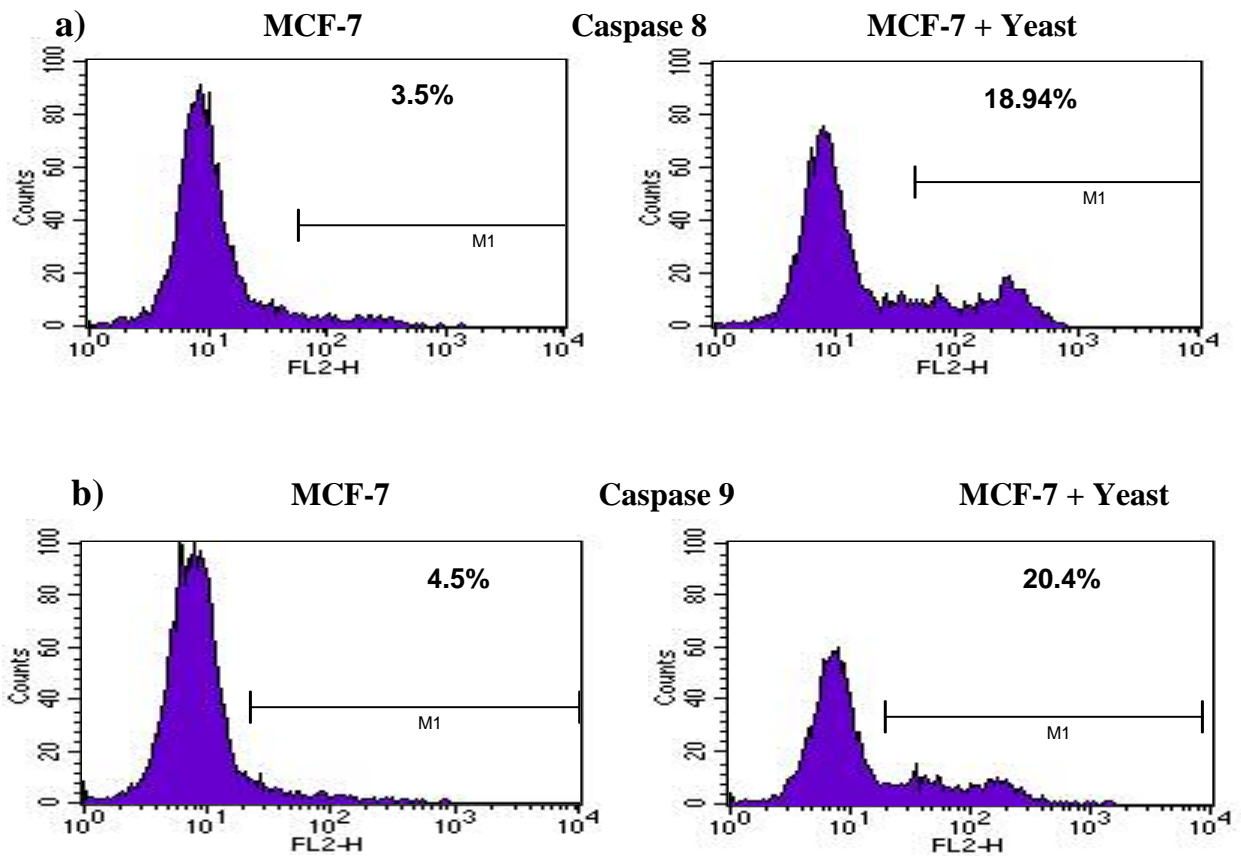
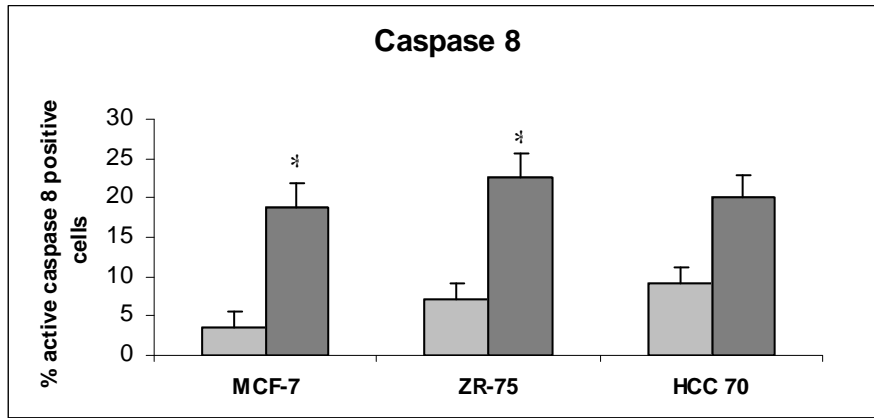
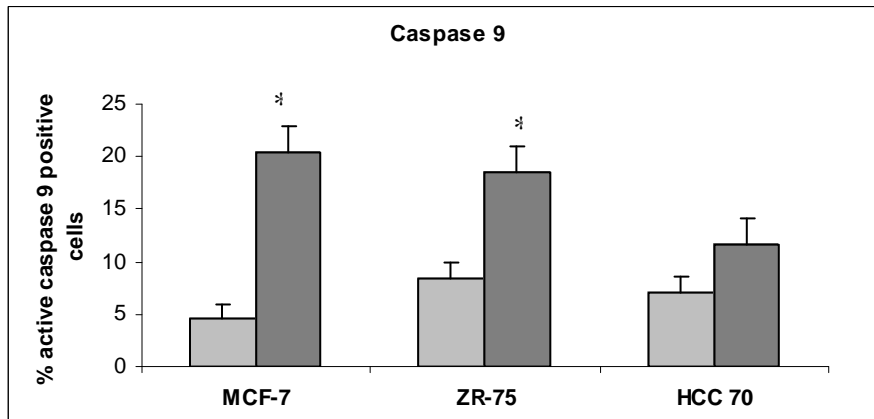


Figure 6

a)



b)



c)

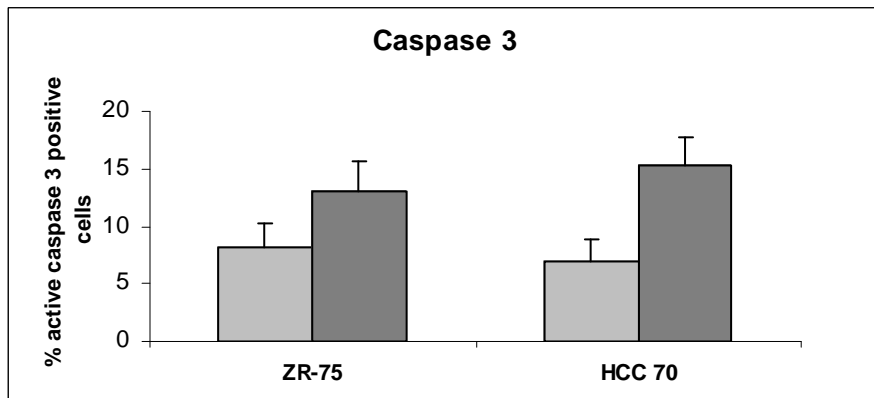


Table I- Apoptosis of BCCs in the presence of caspase inhibitors

Treatment	MCF-7	ZR-75
No Treatment	2.6	7.2
Yeast alone	22	19.9
<i>Yeast + Caspase 8 Inhibitor</i>		
Low Dose	21	26
High Dose	21	23.6
<i>Yeast + Caspase 9 Inhibitor</i>		
Low Dose	22	24
High Dose	22.6	25.6
<i>Yeast + Caspase 3 Inhibitor</i>		
Low Dose	-	23
High Dose	-	24

MCF-7 and ZR-75 cells were treated with caspase 8, 9 and 3 inhibitors. The inhibitors were used at two different concentrations; low dose (1 μ M/ml) and high dose (4 μ M/ml). Cells were then cultured with yeast and apoptosis was determined by flow cytometry.

Figure 7

