Heat shock protein 27 inhibition of apoptosis in human breast cancer cells

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HEAT SHOCK PROTEIN 27 INHIBITION OF APOPTOSIS IN HUMAN BREAST CANCER CELLS

by

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ABSTRACT

Heat Shock Protein 27 Inhibition of Apoptosis in Human Breast Cancer Cells
by Sindhu Padmanabhan

Dr. Stephen W. Carper, Examining Committee Chair
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Cancer occurs when normal cells fail to die in response to various cell death signals. Normal eukaryotic cells exhibit two major forms of cell death – Necrosis and Apoptosis. Apoptosis can be triggered in cells by various agents. Studies have shown that an elevated amount of Hsp 27 protects cells from apoptosis induced by Sodium Butyrate. In this study, we treated estrogen receptor negative human breast cancer cells with apoptotic inducers such as Sodium Butyrate, Staurosporine, Vitamin E Succinate and Cycloheximide. The cells that constitutively expressed Hsp 27 had a higher growth in the presence of Cycloheximide and Vitamin E Succinate and also exhibited lesser apoptosis in the presence of Sodium Butyrate and Staurosporine. This study demonstrates that Hsp 27 blocks cell death in breast cancer cells in the presence of certain cytotoxic drugs and the presence of this protein could serve as a marker for aggressive tumors.
<table>
<thead>
<tr>
<th>TABLE OF CONTENTS</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABSTRACT ................................................................. iii</td>
</tr>
<tr>
<td>LIST OF FIGURES .............................................................. vi</td>
</tr>
<tr>
<td>ACKNOWLEDGEMENTS ......................................................... viii</td>
</tr>
<tr>
<td>CHAPTER 1 INTRODUCTION ...................................................... 1</td>
</tr>
<tr>
<td>Cancer ............................................................. 1</td>
</tr>
<tr>
<td>A Faulty Regulation in Cancer Cells ......................... 1</td>
</tr>
<tr>
<td>Apoptosis as a Biochemical Process ..................... 2</td>
</tr>
<tr>
<td>Small Heat Shock Proteins and Apoptosis .......... 7</td>
</tr>
<tr>
<td>Hsp 27 and Breast Cancer ................................... 8</td>
</tr>
<tr>
<td>Significance of the Study .................................. 9</td>
</tr>
<tr>
<td>CHAPTER 2 MATERIALS AND METHODS ................................. 10</td>
</tr>
<tr>
<td>Cell Lines .......................................................... 10</td>
</tr>
<tr>
<td>Cytotoxic Drugs ..................................................... 10</td>
</tr>
<tr>
<td>Sodium Butyrate ..................................................... 11</td>
</tr>
<tr>
<td>Staurosporine ........................................................ 11</td>
</tr>
<tr>
<td>Vitamin E Succinate .............................................. 12</td>
</tr>
<tr>
<td>Cycloheximide ........................................................ 12</td>
</tr>
<tr>
<td>Growth Assay ........................................................ 12</td>
</tr>
<tr>
<td>Experimental Procedure ..................................... 15</td>
</tr>
<tr>
<td>Apoptosis Assay ...................................................... 15</td>
</tr>
<tr>
<td>Experimental Procedure ..................................... 16</td>
</tr>
<tr>
<td>CHAPTER 3 RESULTS ........................................................ 18</td>
</tr>
<tr>
<td>Growth Inhibition of Breast Cancer Cells in the Presence of Sodium Butyrate 18</td>
</tr>
<tr>
<td>Apoptosis Induced by Sodium Butyrate .................. 19</td>
</tr>
<tr>
<td>Growth Inhibition by Staurosporine .................... 20</td>
</tr>
<tr>
<td>Induction of Apoptosis by Staurosporine ................ 21</td>
</tr>
</tbody>
</table>
LIST OF FIGURES

Figure 1 Ultrastructural Features of Apoptosis and Necrosis .................. 3
Figure 2 Nucleosomes-DNA Associated with Histones ......................... 4
Figure 3 Common Apoptotic Pathway ................................................. 5
Figure 4 Reduction of MTT to Formazan ............................................ 13
Figure 5 Points of Interaction of MTT with Electron Transport Chain .... 14
Figure 6 Double Sandwich ELISA: Test Principle ................................ 17
Figure 7 Percentage Growth of DB 46 and DC 4 Cells 48h after Treatment with Butyrate .......................................................... 25
Figure 8 Percentage Growth of DB 26 and DC 7 Cells 48h after Treatment with Butyrate .......................................................... 26
Figure 9 Percentage Growth of DB 46 and DC 4 Cells 24h after Treatment with Butyrate .......................................................... 27
Figure 10 Percentage Growth of DB 26 and DC 7 Cells 24h after Treatment with Butyrate .......................................................... 28
Figure 11 Fold Increase in Apoptosis of DB 46 and DC 4 Cells 20h after Treatment with Butyrate .......................................................... 29
Figure 12 Fold Increase in Apoptosis of DB 26 and DC 7 Cells 20h after Treatment with Butyrate .......................................................... 30
Figure 13 Percentage Growth of DB 46 and DC 4 Cells 24h after Treatment with Staurosporine ....................................................... 31
Figure 14 Percentage Growth of DB 26 and DC 7 Cells 24h after Treatment with Staurosporine ....................................................... 32
Figure 15 Fold Increase in Apoptosis of DB 46 and DC 4 Cells 24h after Treatment with Staurosporine ....................................................... 33
Figure 16 Fold Increase in Apoptosis of DB 26 and DC 7 Cells 24h after Treatment with Staurosporine ....................................................... 34
Figure 17 Percentage Growth of DB 46 and DC 4 Cells 48h after Treatment with VES .......................................................... 35
Figure 18 Percentage Growth of DB 46 and DC 4 Cells 65h after Treatment with VES .......................................................... 36
Figure 19 Percentage Growth of DB 26 and DC 7 Cells 65h after Treatment with VES .......................................................... 37
Figure 20 Percentage Growth of DB 46 and DC 4 Cells 24h after a 2 hour Treatment with Cycloheximide .......................................................... 38
Figure 21  Percentage Growth of DB 26 and DC 7 Cells 24h after a 2 hour Treatment with Cycloheximide....................................................... 39
Figure 22  Percentage Growth of DB 46 and DC 4 Cells 65h after a 2 hour Treatment with Cycloheximide....................................................... 40
Figure 23  Percentage Growth of DB 26 and DC 7 Cells 65h after a 2 hour Treatment with Cycloheximide....................................................... 41
Figure 24  General Apoptotic Pathway.............................................................. 52
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CHAPTER 1

INTRODUCTION

Cancer

Cancer is a cellular disease characterized by the uncontrolled proliferation of cells leading to a local growth or tumor. Tumors are a caricature of the normal processes of tissue renewal; they arise when the normal processes of growth and differentiation go awry (1). In the normal cells that make up different parts of our body, each cell has a definite function and a predictable life cycle. The growth and division of normal cells is regulated by complex biochemical processes i.e., when a fully functional cell becomes senescent; it is shed and replaced by another cell that takes its place. A cancerous growth results when the regulation of the cell cycle is upset and cells fail to respond to cell death signals.

A Faulty Regulation in Cancer Cells

Normal cells go through a specific number of cell cycles after which they die via a programmed cell death process known as apoptosis. Apoptosis is distinct mode of cell death occurring during the normal processes of growth and
development (2). It is responsible for deletion of cells in normal tissues and is characterized by plasma membrane blebbing, condensation of cytoplasm and nucleus, and cellular fragmentation into membrane bound apoptotic bodies, which are phagocytised by neighboring cells (2-6)(Figure 1, page 3). Biochemically, apoptosis is characterized by extensive endonucleolytic cleavage of double stranded DNA initially into large fragments of 50-300 kilobases (6). The endonucleases then cleave the DNA at linker regions producing nucleosomes, 200 base pairs of DNA wound around a histone octamer (Figure 2, page 4). Cells can also die by necrosis, which involves swelling of cytoplasm and organelles followed by lysis of plasma membrane and random DNA fragmentation (2). An interesting feature of apoptosis is that it can take place in isolated cells and unlike necrosis does not generate an inflammatory response. Apoptosis becomes important in a pathological context too, its deregulation leading to disease conditions such as cancer, neurodegeneration, ischemia, senescence and AIDS (7). Since apoptosis can be induced in cells, knowledge of its mechanism can be a powerful tool in manipulating an apoptotic response in cancer and other diseases.

Apoptosis as a Biochemical Process

Apoptosis can be induced in cells by a variety of agents such as radiation (ionizing and UV), chemotherapeutic drugs, heat, hypoxia, glucocorticoids growth factor deprivation (2)(Figure 3, page 5). The exact mechanism(s)
Figure 1  Diagram illustrating the ultrastructural features of apoptosis and necrosis. (Adapted from Kerr, et al., Cancer, 73:2014, 1994)
Figure 2  Nucleosomes – DNA associated with histones
Triggers → Modulators → Effectors → Death substrates

DNA damage, growth factor withdrawal, hypoxia, heat, glucocorticoids, etc.
p53, bcl-2, kinases ceramide oncogenes transcription factors, cytochrome c, etc.

Cysteine proteases Lamins, PARP, fodrin, etc.

Death

Figure 3 Common Apoptotic Pathway
by which these agents trigger apoptosis is not known but a family of cysteine proteases (related to the interleukin-1β-converting enzyme (ICE)) become activated and execute apoptosis through specific cleavage of substrates known as death substrates (8,9). Cysteine proteases belong to the family of enzymes that degrade proteins. These proteases have a reactive cysteine at its active site (sulphhydryl site), which is responsible for catalyzing the cleavage of peptide bonds.

The cysteine proteases, the major effectors of cell death, are present as inactive precursor polypeptides that are activated during the death process by cleavage at aspartic acid residues (9). These activated proteases cleave the death substrates at specific aspartic acid residues too, and hence are known as cysteine-aspases or Caspases (9,10,11). Recent work has shown that there are eleven mammalian caspases, which are divided into four classes on the basis of their molecular homology (9). The activated caspases go on to cleave (a) DNA repair enzymes such as poly (ADP)-ribose polymerase and DNA dependent protein kinase, DNA-PK, thereby abolishing the DNA repair ability in cells (b) Structural proteins like lamin, leading to nuclear condensation, (c) Cytoskeletal proteins including actin, Gas-2 and fodrin, inducing cell shrinkage and membrane blebbing, (d) Small ribonucleoprotein U1-70kDa, and (e) cell cycle mediators such as protein kinase C δ and retinoblastoma protein Rb (11,12). Recently it has been reported that caspases are also responsible for activating a calcium dependent endonuclease, CAD, responsible for cleaving the DNA at the linker
regions (Figure 2, page 4) between nucleosomes by specifically cleaving and inactivating ICAD, the inhibitor of CAD (13). Hence turning on caspases acts as an irreversible signal for death by apoptosis.

Small Heat Shock Proteins and Apoptosis

Heat shock proteins (Hsp) are a unique family of highly conserved proteins produced by cells in response to environmental and pathophysiological stresses (14,15,16). The heat shock family of proteins is subdivided into four major classes based on the molecular weight of these proteins. They are Hsp 110, Hsp 90, Hsp 70 and Small heat shock proteins (sHsp) which include Hsp 27. Hsp’s are produced in response to environmental stresses such as heat shock, UV radiation, heavy metals and oxidants, physiological stimulants such as hormones, growth factors and cell cycle regulators, and pathophysiological conditions such as infections and glucose starvation (16). The main function of the Hsp 90 and Hsp 70 class of heat shock proteins is molecular chaperoning i.e., to preserve the proteins within the stressed cell in a folded state so that they are not denatured during the stress. In addition to the chaperoning function, these proteins are also involved in signal transduction and thermotolerance. Small heat shock proteins like Hsp 27 have been known to protect cells from oxidative stress, apoptosis and chemotherapeutic drugs (4,5,17).
Hsp 27 and Breast Cancer

In normal breast tissue, the expression of Hsp 27 is very low whereas it is present in very high amounts in human breast tumor cells (18). The presence of this protein is a prognostic marker for more aggressive tumors and indicates shortened disease free survival and increased resistance to chemotherapeutic drugs (19). Hsp 27 is an estrogen responsive protein. Mammary carcinoma cells having receptors for estrogen (ER+) and also expressing Hsp 27 can be sensitized to chemotherapeutic drugs by treating them with estrogen antagonists. Estrogen antagonists are structurally similar to estrogen and bind to the estrogen receptor. The antagonist binding inhibits estrogen from binding to its receptor, decreasing the proliferative effects caused by the binding of estrogen to its receptor. The presence of Hsp 27 in ER+ tumors is believed to increase the rate of proliferation of these cancerous cells (20). However we have seen Hsp 27 protect estrogen receptor negative (ER-) breast cancer cells from cytotoxic drugs and previous work has shown that these ER- cells can also be sensitized to doxorubicin, a chemotherapeutic drug, by the estrogen antagonist toremifene (21). The exact mechanism by which Hsp 27 confers protection to tumor cells against chemotherapeutic drugs is still unclear.
Significance of the Study

The main goal of this work is to understand the role of Hsp 27 in ER- breast cancer cells. The presence of Hsp 27 in ER- breast cancer cells, as shown by our study protects these cells from various cytotoxic drugs. The exact mechanism by which Hsp 27 confers protection is not known, but its ability to do so at various points in the apoptotic pathway leads us to believe that it is acting downstream to these inducers.
CHAPTER 2

MATERIALS AND METHODS

Cell Lines

The cell lines used in this study are derived from the estrogen receptor negative human breast cancer cells MBA-MD-231 by transfection with the constitutive expression vector pβ27 (DB 46 and DB 26) or the control vector pHβApr-1-neo (DC 4 and DC 7) (21). Stable transfected cell lines were isolated and grown in Minimal Essential Media (MEM) (Gibco BRL), supplemented with 10% fetal bovine serum, 25 mM HEPES buffer, 100 IU penicillin/ml, 100µg/ml streptomycin, 2mM L-glutamine (Biowhittaker Inc.), and 600 µg/ml G418 sulfate (Gibco BRL). All cultures were maintained at 37°C in humidified incubators with 5% CO₂ : 95% air. Twenty-four hours prior to an experiment, cells were washed and resuspended in supplemented MEM, lacking G418.

Cytotoxic Drugs

Sodium Butyrate, Staurosporine, Vitamin E Succinate and Cycloheximide

10

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were used in this study because of their reported capacity to act as apoptotic inducers (25-37). Growth inhibition and apoptosis induced by each drug was studied using MTT assay and enzyme linked immuno absorbent assay (ELISA) respectively (described in detail in separate sections). The drug treatment (i.e. the incubation time with the drug) and concentration of the drug varied with the drug used.

Sodium Butyrate

Sodium Butyrate (Sigma Chemical Co.) was prepared in deionized water (100mM). Appropriate concentrations of the drug was added to Hsp 27 expressing and control transfected cells, 24 hours after the cells were plated in a 96 well plate (this incubation is done to let the cells attach to the plate). Growth inhibition was studied 24h and 48h after incubation with the drug. Apoptosis was measured 24h after incubation with the drug.

Staurosporine

Staurosporine (Sigma Chemical Co.) was prepared in DMSO (100 μM). Appropriate concentrations of the drug was added to Hsp 27 expressing and control transfected cells, 24 hours after the cells were plated in a 96 well plate. Growth inhibition and apoptosis was studied 24h after incubation with the drug.
Vitamin E Succinate

Vitamin E Succinate (Sigma Chemical Co.) was prepared in 100% ethanol (5mM-15mM). Succinic acid (Sigma Chemical Co.) prepared in ethanol (1mg/ml) was used as control. Appropriate concentrations of the drug was added to Hsp 27 expressing and control transfected cells, 24 hours after the cells were plated in a 96 well plate. Growth inhibition was studied at 48h and 65h.

Cycloheximide

Cycloheximide (Sigma Chemical Co.) was prepared in 100% ethanol (10mM-40mM). Appropriate concentrations of the drug was added to Hsp 27 expressing and control transfected cells, 24 hours after the cells were plated in a 96 well plate. Various concentrations of Cycloheximide was added at the end of the incubation period and reincubated for 2h. At the end of the incubation period with the drug, 200µl media was removed and 200 µl of fresh media was added to wells and the plate was incubated at 37°C. Growth inhibition was measured 24h-65h later.

Growth Assay

The growth of the control transfected (DC 4 and DC 7) and Hsp27 expressing cell lines (DB46 and DB26) in the presence of cytotoxic drugs was measured using MTT (Sigma Chemical Co.) dye uptake procedure. The MTT
assay is a quantitative colorimetric assay that is rapid and does not involve the use of any radioisotopes (22).

MTT FORMAZAN

Figure 4 Reduction of MTT to Formazan

MTT, (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide), is a water soluble tetrazolium salt yielding a pale yellow solution in media or saline solutions lacking phenol red. The mitochondrial dehydrogenases of living cells take up MTT and metabolize it to formazan, an insoluble purple product (Figure 4).

This conversion takes place in the inner mitochondrial membrane by the enzymes of the electron transfer chain (23). In the presence of MTT, this chain is disrupted and, the electrons are accepted by MTT, which undergoes reduction to formazan (Figure 5). The formazan formed is solubilized using acid isopropanol and measured spectrophotometrically using a plate reader at 570nm. The optical
density directly corresponds to activity of living cells relative to the control (untreated cells).

Figure 5 Points of Interaction of MTT with the Electron Transport Chain

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Experimental Procedure

About $1 \times 10^4$ cells/200\mu l of culture medium were seeded into wells of a 96 well plate and incubated at 37°C for 24h. After 24 h, the medium was changed and appropriate cultures were treated with various cytotoxic drugs and incubated for 24-48h. At the end of the incubation period, 20 \mu l of MTT (5mg/ml) was added and the plate was wrapped in aluminum foil and incubated at 37°C for 4h. After the incubation period, 150 \mu l of the media was removed and 150 \mu l of isopropyl alcohol:1N HCl (98:4) was added and the absorbance determined at 570nm using a microplate reader (Dynatech MR 5000). Results are presented as a percentage of the control.

Apoptosis Assay

Apoptosis was quantitated using an enzyme linked immunosorbent assay (Boehringer Mannheim), which measures the cytoplasmic histone-bound DNA fragments (mono and oligonucleosomes) generated during apoptotic DNA fragmentation (Figure 6, page17). The presence of these fragments in the cytoplasm is a characteristic of apoptosis and is a result of endogenous endonucleases being activated by the proteolytic cascade. Antibodies directed at the DNA and histones in this “sandwich” assay, quantitatively determines apoptotic death induced by the cytotoxic drug relative to the control (untreated

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cells). The two antibodies used in this assay are (1) Anti-histone antibody that targets the histone and (2) Anti-DNA-peroxidase that targets the DNA. The amount of these fragments present in the cytoplasm i.e., the amount of peroxidase in the immunocomplex is determined photometrically by conjugation with ABTS (2,2'-azino-di-[3-ethylbenzthiazolinesulfonate(6)]) as a substrate. The specificity of the antibodies to histone and DNA of the nucleosomes was not studied here, as we were interested in comparing the apoptosis induced in the Hsp 27 expressing cell lines to the control transfected cell line.

**Experimental Procedure**

Cells were plated (1 x 10^4 cells/200 µl) into wells of a 96-well plate. After desired treatment, cytoplasmic extracts were made from both floating and attached cells, according to manufacturer's protocol. Briefly, drug treated cells and untreated controls were lysed using a lysis buffer and centrifuged at 1000 r.p.m. for 10 minutes. Control and drug-treated cell extracts were equalized on the basis of equal cell number. The wells were first coated with anti-histone antibody, loaded with cytoplasmic extracts (centrifugate) and followed by incubation with anti-DNA second antibody conjugated with peroxidase. The ELISA was developed with peroxidase substrate, and the absorbance at 405 nm was measured using microplate autoreader (Dynatech MR5000).
Figure 6  Double sandwich ELISA: Test Principle

Anti histone coated plate  Nucleosomes  Anti DNA peroxidase  ABTS substrate
CHAPTER 3

RESULTS

Growth Inhibition of Breast Cancer Cells in the Presence of Sodium Butyrate

- Sodium butyrate, a short chain fatty acid, is produced in the colon by the fermentation of dietary fiber (24). Sodium butyrate and other short chain fatty acids (SCFA) have been known to induce growth arrest and promote apoptosis in many cell types including breast cancer cells (25-30). Sodium butyrate is nontoxic and hence can be used for therapeutic purposes. In this study, the effect of sodium butyrate on the growth of breast cancer cells expressing Hsp 27 (DB 46 and DB 26) was investigated and compared to their control transfected counterparts which do not express Hsp 27 (DC 4 and DC 7). All cell lines were treated continuously with various concentrations of sodium butyrate for 24 h or 48 h. It has been reported that sodium butyrate causes growth arrest in the first 48 h, continuous treatment beyond this point led to little additional reduction in cell survival (26). The growth inhibition was measured using the MTT assay as described in Materials and Methods. As shown in Figure 7, (page 25), DC 4 cells were more sensitive to sodium butyrate at 48 h (46% growth at the highest concentration of butyrate) than DB 46 cells (54% growth at the highest
concentration of butyrate). These results are from a single experiment performed in triplicate. The experiment was repeated with DB 26 and DC 7 cells (Figure 8, page 26). In this case however, the growth of Hsp27 expressing cells (DB 26) was not significantly higher than control transfected cells (DC 7). The validity of the results were tested by using a t-test (Appendix). The error bars correspond to percentage deviation from the mean. At 24 h, however, the growth inhibition by butyrate was lower, and any differences between the cell lines were not statistically significant (Figure 9, page 27 and Figure 10, page 28).

Apoptosis Induced by Sodium Butyrate

The ability of Sodium Butyrate to induce apoptosis in our cell lines was studied using the enzyme linked immunosorbent assay (ELISA) as described in Materials and Methods. As shown in Figure 11 (page 29), Sodium Butyrate treatment of DB 46 and DC 4 for 20 hours stimulated apoptosis in a dose dependent manner. The addition of 10 mM Sodium Butyrate was able to increase the rate of apoptosis in the control transfected cell line (DC 4) by almost 6.5 fold while in Hsp27 expressing cell line apoptosis was increased only 2 fold. These results are from a single experiment performed in triplicate. DB 26 cells however did not exhibit a dramatic resistance to butyrate (Figure 12, page 30). These cells exhibited higher apoptosis in the presence of 3mM Sodium Butyrate (5.0 fold increase in apoptosis) than the DB 46 cells (2.0 fold increase in apoptosis with
respect to control). The differences in the Hsp 27 expressing cell lines (i.e., in their response to butyrate) may be due to clonogenic variations (i.e., the exact amount of the protein present in different clones may be different).

Growth Inhibition by Staurosporine

Staurosporine, a microbial alkaloid induces its anti-cancer activity by the inhibition of protein kinase C (31). Protein kinases are a group of enzymes that catalyze the phosphorylation of target proteins and regulate protein activity. Protein kinase C, a calcium dependent protein kinase, phosphorylates proteins at their serine and threonine residues. Diacylglycerol and tumor promoters such as phorbol esters activate this enzyme. Phorbol esters are structurally similar to diacylglycerol but are not easily metabolized. Since protein kinase C has been implicated in cellular proliferation (32), a persistent activation of this enzyme by phorbol esters leads to the formation of tumors. Staurosporine is a potent protein kinase C inhibitor (31) and induces growth arrest and apoptosis in a variety of cell lines (33-35).

To determine whether Hsp 27 could offset the anti-proliferative and apoptotic potential of Staurosporine, we treated Hsp 27 expressing and control transfected breast cancer cells with micromolar amounts of Staurosporine for a period of 24h. Our results indicate that at the highest concentration (5\(\mu\)M), Staurosporine had 93%-96% inhibition of growth, as studied by the MTT assay.
The Hsp 27 expressing cell lines did not have a higher tolerance for the drug when compared to the control transfected cell lines. Any differences seen between the cell lines were not statistically significant (Figure 13, page 31 and Figure 14, page 32). Each experiment was performed in triplicate and the error bars represent the percent deviation from the mean.

**Induction of Apoptosis by Staurosporine**

- Apoptosis was quantified using an ELISA that measures cytoplasmic histone-bound DNA complexes generated during apoptotic DNA fragmentation. As shown in Figure 15 (page 33), Staurosporine induced apoptosis in a dose dependent manner in DB 46 cells whereas the DC 4 cells apoptosis increased eleven fold for the lowest concentration (0.5μM) and higher concentrations had little effect in increasing apoptosis. At the highest concentration used, the protection offered by Hsp 27 in DB 46 cells no longer existed, in fact at this concentration DB 46 cells had higher apoptosis than DC 4 cells. This result is important therapeutically, as it tells us that higher concentrations of the drug may be used to treat patients having Hsp 27 positive tumors. Figure 16 (page 34) shows the effect of the drug on DB 26 and DC 7 cell lines. However, DC 7 (which is similar to DC 4) shows a dose response at all concentrations of the drug used. At all concentrations of the drug used DB 26 cells exhibited lower apoptosis than the control transfected cells DC 7. The dramatic sensitivity seen in DC 4 cells at lower concentrations of the drug (11 fold increase in apoptosis at 0.5
µM) was not seen in DC 7 cells (only 4 fold increase in apoptosis at 0.5 µM). Such differences may be attributed to clonogenic variations.

Growth Inhibition by Vitamin E Succinate

Vitamin E Succinate (RRR-α-tocopheryl succinate) is the succinate ester of Vitamin E. The succinate ester has been demonstrated to be a potent inhibitor of human breast cancer cell proliferation (36). The exact mechanism by which Vitamin E Succinate (VES) inhibits proliferation is not known, but cell cycle blockages, cellular differentiation, activation of transforming growth factor-β and induction of apoptosis is seen in VES treated cells. VES is mainly attractive for its non-toxic effect on normal cell types. Since a lot of recent work has focused on the anti-tumor effect of VES in breast cancer cells, we were interested in knowing whether VES could exhibit the same effect or activity in Hsp 27 expressing breast cancer cells. In this study, we exposed Hsp 27 expressing and control transfected cell lines to various concentrations of VES continuously for 48-65 h. After 48 h of incubation, 90%-93% growth inhibition was seen at 40 µg/ml of VES (Figure 17, page 35). When cells were exposed to 5 µg/ml - 30 µg/ml of VES for 65 h (Figure 18, page 36 and Figure 19, page 37), they showed a similar response. The highest growth inhibition seen for all four cell lines was at 15 µg/ml. At all concentrations, however, Hsp 27 expressing cell lines had higher tolerance for the drug than the control transfected cell lines. The
growth inhibition at various concentrations was normalized to a control, which had succinic acid at the same concentration as the highest concentration of VES used. At 65h, the growth inhibition seen in the presence of VES was higher than the growth inhibition seen for the same concentrations at 48 h.

Growth Inhibition by Cycloheximide

Cycloheximide is an antibiotic that inhibits protein biosynthesis. The exact mechanism as to how the inhibition of protein synthesis turns on the apoptotic cascade is not known. An explanation that has been put forward is that Cycloheximide blocks the synthesis of inhibitor proteins that regulate the endogenous endonucleases (37). When the levels of the inhibitor proteins fall, the endonucleases are activated and apoptosis follows. It has also been reported that some cells are programmed to die in the absence of inhibitor proteins (38). In our study, the effect of Hsp 27 on growth inhibition induced by Cycloheximide was studied using the MTT assay. Human breast cancer cells that constitutively express Hsp 27 and their control transfected counterparts were treated with 25μM-200μM of Cycloheximide for a period of two hours and then allowed to grow for 24h-65h. Figure 20 (page 38) and Figure 21 (page 39) shows the percentage growth of cells 24h after the Cycloheximide treatment. The growth in both cases was normalized to a control that was treated with 0.1%v/v with ethanol. The cells that expressed Hsp 27 showed a higher percentage of growth than the control transfected cell lines at all concentrations of the drug studied.
The error bars were the percent deviation from the mean of the experiment set in triplicate. Figure 22 (page 40) and Figure 23 (page 41) show the effect of two-hour pulse of Cycloheximide studied after 65 h. The DB 26 cells (Figure 23) did not show a significantly higher growth when compared to the control transfected DC 7 cells. In all other cases, (Figures 20-22) Hsp 27 expressing breast cancer cells had a higher resistance to the drug than their control transfected counterparts.
Figure 7  Percentage growth of DB 46 and DC 4 cells 48h after treatment with butyrate. The error bars represent percent deviation from mean (n=3). (*: p<0.05). OD for untreated controls: (DB 46: 0.461 and DC 4: 0.451)
Figure 8  Percentage growth of DB 26 and DC 7 cells 48h after treatment with butyrate. O.D. for untreated controls: (DB 26: 0.474 and DC 7: 0.347). (*: p=0.05)
Figure 9  Percentage growth of DB 46 and DC 4 cells 24h after treatment with butyrate. The error bars represent percent deviation from mean (n=3). (• : p=0.05). O.D. for untreated controls: (DB 46: 0.334 and DC 4: 0.275)
Figure 10  Percentage growth of DB 26 and DC 7 cells 24h after treatment with butyrate. The error bars represent percent deviation from mean (n=3). (∗ : p<0.05). O.D. for untreated controls: (DB 26: 0.305 and DC 4: 0.254)
Figure 11  Fold increase in apoptosis of DB 46 and DC 4 cells 20h after treatment with butyrate. The error bars represent standard deviation from mean (n=3). (• : p<=0.05). O.D. for serum control:0.364
Figure 12 Fold increase in apoptosis of DB 26 and DC 7 cells 24h after treatment with butyrate. The error bars represent standard deviation from mean (n=3). (• : p = 0.05). O.D. for serum control: 0.313
Figure 13  Percentage growth of DB 46 and DC 4 cells 24h after treatment with Staurosporine. The error bars represent percent deviation from mean (n=3). (♦ too few data values to determine statistical significance). O.D. for untreated controls: (DB 46: 0.272 and DC 4: 0.288)
Figure 14  Percentage growth of DB 26 and DC 7 cells 24h after treatment with Staurosporine. The error bars represent percent deviation from mean (n=3). (•: p <=0.05). O.D. for untreated controls:(DB 26: 0.278 and DC 7: 0.214)
Figure 15  Fold increase in apoptosis of DB 46 and DC 4 cells 24h after treatment with Staurosporine. The error bars represent standard deviation from mean (n=3). (• : p <= 0.05). O.D. for serum control: 0.240
Figure 16  Fold increase in apoptosis of DB 26 and DC 7 cells 24h after treatment with Staurosporine. The error bars represent standard deviation from mean (n=3). O.D. for serum control: 0.240.
Figure 17  Percentage growth of DB 46 and DC 4 cells 48 h after treatment with VES. The error bars represent percent deviation from mean (n=3). (.*, p=0.05). O.D. for untreated controls: (DB 46: 0.407 and DC 4: 0.325)
Figure 18  Percentage growth of DB 46 and DC 4 cells 65h after treatment with VES. The error bars represent percent deviation from mean (n=3). (* : p <=0.05). O.D. for untreated controls: (DB 46: 0.609 and DC 4: 0.551)
Figure 19  Percentage growth of DB 26 and DC 7 cells 65h after treatment with VES. The error bars represent percent deviation from mean (n=3). (* : p <=0.05). O.D. for untreated controls:(DB 26: 0.638 and DC 4: 0.498)
Figure 20 Percentage growth of DB 46 and DC 4 cells 24h after a 2 hour treatment with Cycloheximide. The error bars represent percent deviation from mean (n=3). (♦ : p<=0.05). O.D. for untreated controls:(DB 46: 0.184 and DC 4: 0.202)
Figure 21    Percentage growth of DB 26 and DC 7 cells 24h after a 2 hour treatment with Cycloheximide. The error bars represent percent deviation from mean (n=3). (*: p <= 0.05). O.D. for untreated controls: (DB 26: 0.225 and DC 7: 0.461)
Figure 22  Percentage growth of DB 46 and DC 4 cells 65h after a 2 hour treatment with Cycloheximide. The error bars represent percent deviation from mean (n=3). (• : p <= 0.05). O.D. for untreated controls:(DB 46: 0.426 and DC 4: 0.511)
Figure 23 Percentage growth of DB 26 and DC 7 cells 65h after a 2 hour treatment with Cycloheximide. The error bars represent percent deviation from mean (n=3). (• : p < 0.01). O.D. for untreated controls:(DB 26: 0.631 and DC 7: 0.473)
Hsp 27 protects the cells from stresses such as heat shock and chemotherapeutic agents (15-17). This study focuses on yet another aspect of the protection offered by this protein - its ability to inhibit programmed cell death or apoptosis. It has been reported that the presence of Hsp 27 in breast cancer cells has correlated to a shorter disease free survival and as a marker for more aggressive tumors (19). In order to understand the extent of protection offered by Hsp 27 against various cytotoxic drugs, we used human breast cancer cells that constitutively express Hsp 27. These cells were constructed from the estrogen receptor negative human breast cancer cell line MDA-MB-231 which expresses very low endogenous levels of Hsp 27. MDA-MB-231 cells were transfected with the constitutive expression vector pβ27 containing the human Hsp 27 cDNA sequence or with a control vector pHβApr-1-neo lacking the cDNA sequence (21). The constitutive expression of Hsp 27 has been reported to protect breast cancer cells from heat induced cytotoxicity and also decrease the time required to resume protein synthesis (17). Others have reported a similar finding using different cell lines.
One of the methods that Hsp 27 may confer protection is by inhibiting cell death – either necrosis or apoptosis. Since many of the chemotherapeutic drugs work by inducing apoptosis, we treated our cells with various cytotoxic drugs which have been reported to induce apoptosis in other cell lines, to see whether Hsp 27 could offset the drug’s effect. We measured the growth inhibition caused by the drug, at first, as an inexpensive indicator of apoptosis and later measured apoptosis induced in some of the systems using an ELISA. The significance of the data obtained was measured using a t-test (Appendix).

In some cases studied, breast cancer cells expressing Hsp 27 had a higher growth in the presence of the cytotoxic drugs. Each of the drugs chosen induced apoptosis in other cell lines by different mechanisms (Figure 24, page 52). In order to have a fair understanding of the point(s) at which Hsp 27 may be acting in the apoptotic cascade to inhibit the onset of apoptosis, I wish to treat each drug separately.

Hsp 27 Confers Resistance against Butyrate Induced Apoptosis

Sodium Butyrate is a dietary micronutrient produced by the microbial fermentation of fiber. Sodium Butyrate and other short chain fatty acids have been reported to induce growth arrest, differentiation and apoptosis in colonic cancer and breast cancer cell lines (25-30). The exact mechanism of butyrate induced apoptosis and growth arrest is not yet known. It has been reported that
butyrate's action is tightly linked to mitochondrial function (27,28). Inhibition of mitochondrial function, by using inhibitors that blocked the mitochondrial electron transport, oxidative phosphorylation and cytochrome c, within 8h of butyrate treatment decreased butyrate induced apoptosis in colonic cancer cells (28). The growth arrest occurs simultaneously at the G_{0}-G_{1} and G_{2}-M of the cell cycle in colonic cancer cells. Breast cancer cells, irrespective of their steroid receptor profiles, were blocked at the G_{2}-M phase of the cell cycle (29). In the same study by Coradini et al. however, only estrogen receptor positive MCF 7 and T47D underwent apoptosis in the presence of butyrate as detected by gel electrophoresis. We observe that the estrogen receptor negative breast cancer cells also undergo apoptosis within 24h of treatment with Sodium Butyrate. The difference in the results may be because of the method used in detecting apoptosis. We used the ELISA system that measures the cytoplasmic histone bound DNA fragments generated during apoptosis, a method that may detect DNA fragmentation even when the classic ladder pattern is not seen on the agarose gel. It has also been reported that butyrate functions as an apoptotic inducer by blocking histone deacetylase in Jurkat lymphoid, colorectal and colon cancer cells (26, 27). When the function of histone deacetylase is inhibited, histone (H4) is hyperacetylated leading to a more open chromatin structure, which opens up the linker regions to endogeneous endonuclease (27). Apoptosis induced in these cell lines is strictly dependent on protein synthesis and activation of caspase 3 and is dependent on a cytochrome c dependent pathway (27) (see
The effect of butyrate induced apoptosis is regulated by Bcl-2, a protein that inhibits apoptosis by inhibiting the transport of cytochrome c from mitochondria to the cytosol (25).

In our study, we observe that human breast cancer cells that express Hsp 27 underwent less apoptosis in the presence of butyrate. Since butyrate functions at the level of mitochondria, Hsp 27 may be functioning at the same level or downstream of mitochondria to offset the effect of Sodium Butyrate. Since induction of apoptosis by butyrate is dependent on cytochrome c dependent pathway which is blocked by bcl-2, it might be interesting to look at whether Hsp 27 has any effect on the release of cytochrome c from the mitochondria.

Hsp 27 Confers Resistance against Staurosporine

Staurosporine, a microbial alkaloid is an inhibitor of a wide range of protein kinases including protein kinase C, cyclin dependent kinases(cdks), cyclic AMP dependent kinases and p60 v-src protein tyrosine kinase. Protein kinases regulate an array of functions, one of which is progression through cell cycle by a family of cdks. The exact function of protein kinase C (PKC) has been addressed previously (see page 20). It has been reported that Staurosporine causes growth arrest at the G1 phase of the cell cycle in breast cancer cells and HeLa cells (33). The growth arrest has been reported to result from an induction of p21 protein and also due to the loss in catalytic activity of the cdks (33,39). p21 is a potent
inhibitor of cdks and associates with various cyclin-cdk complexes to inhibit the kinase activity of these complexes. Staurosporine and its analogues have been reported to alter the cellular localization of certain PKC isoforms and inhibit their activity (32,35). Staurosporine and its hydroxy derivative, however, have been known to activate one of the PKC isoforms, the PKC α. This activation has been reported to occur downstream from caspases in myeloid leukemia cells as inhibitors of caspases blocked the activation of PKC α (35). In other studies, with cardiomyocytes, it was found that Staurosporine activates caspase 3. This activation of caspase 3 may be upstream of the activation of PKC α. It has also been reported that caspase 3 cleaves PKC δ and that this is responsible for the morphological features associated with apoptosis (40).

In our study, estrogen receptor negative breast cancer cells that constitutively express Hsp 27 lower apoptosis than the control transfected cell lines (Figure 15). At the highest concentration of Staurosporine used however, the protection was not observed. This may be because 5μM Staurosporine is highly cytotoxic and offsets the protection offered by Hsp 27. Constitutive expression of Hsp 27 also confers resistance to apoptotic cell death induced by Staurosporine in murine L929 cells. Ongoing research in our labs indicate that Hsp 27 expressing breast cancer cells exhibit lower caspase 3 activity in the first 8h of treatment with cytotoxic drugs than the control transfected cell lines (Joe Stafford, personal communications). This suggests that Hsp 27 may be conferring resistance to Staurosporine by blocking the activation of caspase 3. Higher
concentrations or longer incubation times with the drug may abrogate this effect as caspase 3 showed peak activity after 24h of incubation with cytotoxic drugs.

Hsp 27 Confers Resistance against Vitamin E Succinate

Vitamin E Succinate has been studied widely for its ability to induce growth arrest and apoptosis in a variety of cell lines. The exact mechanism by which VES induces apoptosis is not fully known. Recent reports on the effect of VES in breast cancer cells have shown that VES enhances the expression of transforming growth factor-β (TGF-β) and its isoforms (36,41,42). TGF-β and its isoforms form a family of growth factors that inhibit the proliferation of epithelial, lymphoid and endothelial cells. Malignant transformation has been thought to occur when cells escape the growth-inhibitory effects of TGF-β by either inactivation of the genes encoding for the factor or its receptor. Compounds that stimulate the TGF-β have included retinoids, deltanoloids (Vitamin D and its analogues), tamoxifen (antiestrogen) and gestodene (synthetic progestin)(36). Recently, VES has been added to the list of these terpene compounds that can stimulate TGF-β (42). It has also been reported that VES elevates the levels of c-jun (mRNA and protein) and enhances activator protein-1 (AP-1) binding in a variety of cell lines (42). c-jun is an important member in the AP-1 transcription factor family that has been implicated in cell proliferation, differentiation, transformation and recently in apoptosis. c-jun can be transiently

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activated by growth factors to increase proliferation in cells.

c-jun is activated by the growth factors through the JNK pathway (Jun N terminal kinase) also known as the SAPK (stress activated protein kinase) pathway to cause an increase in proliferation. When JNK is triggered by apoptotic inducers it turns on c-jun by phosphorylation at its serine residue and causes cell death via apoptosis. How the same signaling mechanism causes opposing fates in cells is unknown. One theory is that sustained JNK activation (as seen in the case of apoptotic inducers) induces apoptosis while transient activation (as seen in the case of growth factors) induces proliferation(42).

We observed growth inhibition in breast cancer cells in the presence of VES. The growth inhibition seen in the case of Hsp 27 expressing cells was higher than that seen in the non Hsp 27 expressing cells. Apoptosis was measured at 48h and 65h by an ELISA (data not shown) A significant induction in apoptosis was not seen at times and concentrations of the drug used.

Hsp 27 Confers Resistance against Cycloheximide

Cycloheximide also known as actidone is a glutarimide antibiotic that inhibits protein synthesis in systems that use 80S ribosomes (43). Cycloheximide has been know to induce apoptosis in human breast cancer cells (37). Apoptosis is caused in these cells by the inhibition of protein synthesis. Apoptosis may be turned on when the inhibitor proteins that regulate endonucleases are not
produced in sufficient amounts due to blocked protein synthesis. Cycloheximide has also been reported to interfere with glucose transport which in turn influences cell death. The exact nature of this effect is not yet elucidated. Contrary to its pro-apoptotic function, Cycloheximide has been recently reported to inhibit apoptosis in systems that require new protein synthesis to activate caspases (44). This discrepancy in its function is still not understood.

In human breast cancer cells, Cycloheximide induces a growth inhibition at all concentrations used. However, cells expressing Hsp 27 were able to better survive at all concentration of the drug than the control transfected counterparts. Apoptosis was measured using ELISA at 48 h and 65h, but no induction of apoptosis was seen at the times and concentrations of the drug used (data not shown).

Conclusions

We report that Hsp 27 when present in human breast cancer cells protects these cells from a variety of cytotoxic inducers acting at different points of the apoptotic pathway (see Table 1, page 51). Constitutive expression of Hsp 27 has been reported to protect murine cells from Fas/APO-1 induced apoptosis, U937 and Wehi-s cells from camptothecin, etoposide and actinomycin D and human breast cancer cells from heat shock (4,5,21). Since Hsp 27 can protect different cells from a variety of stresses that induces apoptosis, it might be acting
downstream from these inducers or may be acting at different points in the apoptotic pathway. It has also been reported that Hsp 27 expression decreased intracellular – reactive oxygen species and increase the cellular content of the antioxidant glutathione(4). The presence of the antioxidant glutathione appears to be essential for the protective activity against TNF α and oxidative stress induced cell death. The stimulation of anti-oxidants may be the way in which Hsp 27 protects cells from apoptosis. It has also been reported that Hsp 27 is an actin cap binding protein(45). Actin is one of the substrates cleaved by caspases when apoptosis is turned on. Protection of actin from caspases may be responsible for the anti-apoptotic function of Hsp 27. A clear knowledge of the role of Hsp 27 is not known, the knowledge of its function and the point(s) at which it acts would be crucial in developing adjuvant therapies that target this protein in cancer cells.
Table 1  Summary of Results

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>Drug</th>
<th>Time (h)</th>
<th>Results*</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Growth</td>
<td>Apoptosis</td>
</tr>
<tr>
<td>DB 46 &amp; DC 4</td>
<td>NAB</td>
<td>48 h</td>
<td>✓</td>
<td></td>
</tr>
<tr>
<td>DB 26 &amp; DC 7</td>
<td>NAB</td>
<td>48 h</td>
<td>❖</td>
<td></td>
</tr>
<tr>
<td>DB 46 &amp; DC 4</td>
<td>NAB</td>
<td>24 h</td>
<td>❖</td>
<td>✓</td>
</tr>
<tr>
<td>DB 26 &amp; DC 7</td>
<td>NAB</td>
<td>24 h</td>
<td>❖</td>
<td>❖</td>
</tr>
<tr>
<td>DB 46 &amp; DC 4</td>
<td>STR</td>
<td>24 h</td>
<td>♦</td>
<td>❖</td>
</tr>
<tr>
<td>DB 26 &amp; DC 7</td>
<td>STR</td>
<td>24 h</td>
<td>✓</td>
<td>❖</td>
</tr>
<tr>
<td>DB 46 &amp; DC 4</td>
<td>VES</td>
<td>48 h</td>
<td>❖</td>
<td>O</td>
</tr>
<tr>
<td>DB 46 &amp; DC 4</td>
<td>VES</td>
<td>65 h</td>
<td>✓</td>
<td>O</td>
</tr>
<tr>
<td>DB 26 &amp; DC 7</td>
<td>VES</td>
<td>65 h</td>
<td>❖</td>
<td>O</td>
</tr>
<tr>
<td>DB 46 &amp; DC 4</td>
<td>CHX</td>
<td>24 h</td>
<td>❖</td>
<td></td>
</tr>
<tr>
<td>DB 26 &amp; DC 7</td>
<td>CHX</td>
<td>24 h</td>
<td>❖</td>
<td></td>
</tr>
<tr>
<td>DB 46 &amp; DC 4</td>
<td>CHX</td>
<td>65 h</td>
<td>❖</td>
<td>O</td>
</tr>
<tr>
<td>DB 26 &amp; DC 7</td>
<td>CHX</td>
<td>65 h</td>
<td>❖</td>
<td>O</td>
</tr>
</tbody>
</table>

*: {✓: Significant difference seen in growth between the two cell lines at all concentrations, ❖: differences between the two cell lines significant only at certain concentrations, ♦: too few data values to evaluate the statistical significance, ❖: no significant differences between cells, O: apoptosis not induced at the times and concentration studied, NAB: butyrate, STR: Staurosporine, VES: Vitamin E Succinate, CHX: Cycloheximide}
Figure 24  General Apoptotic Pathway
APPENDIX

CALCULATION OF STATISTICAL SIGNIFICANCE

In order to determine the statistical significance between the growth rates and apoptosis of the DB cell lines (which constitutively express Hsp 27) and DC cell lines (which lack the constitutive expression vector), the student's t-test is used. The student's t-test is generally used to test the hypotheses for mean difference between a set of paired experimental results. In this study all the cell lines were derived from estrogen receptor negative MDA-MB-231 human breast cancer cells. The only difference between the DB cells lines and the DC cell lines is the presence of Hsp 27 cDNA sequence. Hence we considered the DB cell lines and DC cell lines as two sets of samples and any significant difference between their growth rates and apoptosis is attributed to the presence of Hsp 27 in DB cell lines.

This statistical hypotheses is tested assuming normality in the data values and the two mean values are independent from each other with unknown and equal variances. The equation used is given below:

53
\[ t = \frac{\bar{x}_1 - \bar{x}_2}{s_p \sqrt{\frac{1}{n_1} + \frac{1}{n_2}}} \]  

(1)

where

\( n_1 \) = number of observations comprising the first average \( \bar{x}_1 \),

\( n_2 \) = number of observations comprising the second average \( \bar{x}_2 \),

\( s_p \) = pooled standard deviation of the standard deviations \( s_1 \) and \( s_2 \) for the first and second sets of data

\[ s_p = \sqrt{\frac{(n_1 - 1)s_1^2 + (n_2 - 1)s_2^2}{n_1 - 1 + n_2 - 1}} \]  

(2)

The \( t \) value obtained from this calculation is compared with the \( t_p \) given in Table 4, Appendix III (Introduction to Probability and Statistics, by Mendenhall and Beaver, 1990). The \( p \) value determines the significance level. If the \( t \) value exceeded the \( t_p \) value, then the hypotheses is rejected because the mean values of the two samples are significantly different at \( p \) value specified.

In our study, the growth rates of the DB and DC cell lines in the presence of the drug is normalized to an untreated control before performing the t-test. This normalization prevents the differences (growth rates, survival etc., ) between the cell lines from affecting the results seen.
Sample Calculation

A sample calculation is presented to demonstrate the methodology adopted for calculating the statistical significance of the experimental data. The primary objective for using the t-test was to determine if Hsp 27 expressing cell lines were significantly different from control transfected cell lines in their response to various drugs. The results from the following example is presented in Figure 7.

DB 46 is the Hsp 27 expressing breast cancer cell line and DC 4 is the Control transfected cell line. The following data is the O.D. values as measured by the plate reader. The average, standard deviation (SD), % deviation (% SD) and % growth (%G) were calculated manually.

<table>
<thead>
<tr>
<th></th>
<th>DB 46 control</th>
<th>DC 4 control</th>
<th>DB 46 1mM</th>
<th>DC 4 1mM</th>
<th>Raw data as read by the plate reader</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.416</td>
<td>0.414</td>
<td>0.401</td>
<td>0.363</td>
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<td></td>
</tr>
<tr>
<td>0.483</td>
<td>0.452</td>
<td>0.424</td>
<td>0.332</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.489</td>
<td>0.476</td>
<td>0.437</td>
<td>0.347</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.455</td>
<td>0.463</td>
<td>0.432</td>
<td>0.350</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.461</td>
<td>0.451</td>
<td>0.424</td>
<td>0.348</td>
<td></td>
<td>Average</td>
</tr>
<tr>
<td>0.033</td>
<td>0.027</td>
<td>0.016</td>
<td>0.013</td>
<td></td>
<td>SD</td>
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<tr>
<td>100.00</td>
<td>100.00</td>
<td>91.86</td>
<td>77.16</td>
<td></td>
<td>% G</td>
</tr>
<tr>
<td>7.230</td>
<td>5.917</td>
<td>3.761</td>
<td>3.657</td>
<td></td>
<td>% SD</td>
</tr>
</tbody>
</table>

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Calculation of % Growth and % Deviation

The growth of untreated controls was set to a hundred percent and all the drug treated cells were normalized to this control.

i.e.,

For DB 46 cells, the untreated control had an average O.D. of 0.461. Growth was measured by the MTT assay where the sample O.D. corresponds to amount of living cells. Hence O.D. of 0.461 was set to 100% growth and drug treated DB 46 cells were compared to this control.

For DC 4 cells, the untreated control had an O.D. of 0.451 which was set to 100%. The drug treated DC 4 cells were normalized using this value. Therefore,

% growth of DB 46 cells in the presence of 1mM Sodium Butyrate is:

\[ \frac{0.424}{0.461} \times 100 = 91.86 \%
\]

Similarly, % growth of DC 4 cells in the presence of 1mM Sodium Butyrate is:

\[ \frac{0.348}{0.451} \times 100 = 77.16 \%
\]

The percent deviation was calculated using the following equation

% SD = (Standard deviation/Average)\times 100

All other data points in Figure 7 and all other figures have been treated in the same fashion.
Calculation of t

t was calculated using equations (1) and (2). Taking the same example, as before,

<table>
<thead>
<tr>
<th>Raw Data</th>
<th>Normalized data</th>
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</thead>
<tbody>
<tr>
<td>DB46 cont.</td>
<td>DB46 1mM</td>
</tr>
<tr>
<td>DC4 cont.</td>
<td>DC4 1mM</td>
</tr>
<tr>
<td>0.416</td>
<td>0.401</td>
</tr>
<tr>
<td>0.483</td>
<td>0.424</td>
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<td>0.489</td>
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<td>0.432</td>
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<tr>
<td>Average</td>
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<tr>
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<td>0.451</td>
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<td>0.451</td>
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<td>Standard Deviation</td>
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</tr>
<tr>
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<tr>
<td>0.033</td>
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<td>0.027</td>
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<tr>
<td>0.033</td>
<td>0.027</td>
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<tr>
<td>Number of degrees of freedom (n1 and n2)</td>
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<tr>
<td>4.00</td>
<td>4.00</td>
</tr>
<tr>
<td>Pooled standard deviation (s_p)</td>
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</tr>
<tr>
<td>0.030</td>
<td></td>
</tr>
<tr>
<td>t</td>
<td>6.592</td>
</tr>
</tbody>
</table>

As described previously, the drug treated cells of each cell line was normalized to its respective untreated controls. The t test was performed on the
normalized data values and not the raw data. The calculated value of t was compared to accepted values of t at 90%-99% confidence levels. If the calculated value of t was greater than accepted values of t at the confidence levels used, the averages (of the cell lines compared) were significantly different from each other.
BIBLIOGRAPHY


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Thesis Title:  HSP 27 Inhibition of Apoptosis in Human Breast Cancer Cells

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